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ROLE OF MDR3 IN DRUG-INDUCED CHOLESTATIC LIVER INJURY

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To my family

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Abstract

Bile formation is an important physiological process, coordinated by the concerted activity of transmembrane proteins, localized at the canalicular membrane of hepatocytes, belonging to the superfamily of ABC (ATP binding cassette) transporters. These transporters move the primary bile constituents into the canaliculus, a tight intercellular space between apical membranes of adjacent hepatocytes. BSEP (bile salts export pump) pumps bile salts; while MDR3 (multidrug resistance protein 3) and ABCG5/G8 translocate phosphatidylcholine and cholesterol, respectively, making them available for extraction by bile salts. Dysfunctions in the biliary bile salt, phospholipid and cholesterol secretion lead to cholestasis or result in cholesterol crystallization followed by cholelithiasis (gallstone disease). Several drugs may lead to cholestasis by functionally impairing the activity of canalicular transporters. While drug-induced cholestasis due to the inhibition of BSEP is a well investigated mechanism of acquired intrahepatic cholestasis, limited information on the interaction of drugs with lipid transporters such as MDR3 exists, and the role of MDR3 in the pathogenesis of drug-induced cholestasis is poorly understood. The aim of this project was to study the interaction of drugs in a newly established polarized cell line system that expresses the key players involved in canalicular secretion. In the first part of this work, the model LLC-PK₁ cell line stably transfected with human NTCP (Na⁺-taurocholate cotransporting polypeptide), BSEP, MDR3 and ABCG5/G8 and grown in the Transwell[®] system was functionally characterized. The established cell line was shown to display vectorial bile salt transport and specific phosphatidylcholine secretion into the apical compartment. Hence this model system simulates the *in vivo* situation at the canaliculus. Subsequently, using the PC derivative C₆-NBD-PC an assay for testing the effect of compounds on MDR3 activity was established and validated. In the second part of this work, several hepatotoxic as well as potential hepatoprotective (e.g. beneficial on biliary secretion or on bile ducts) drugs were tested in our model system. Of the several hepatotoxic drugs tested, the antifungal azoles, posaconazole, itraconazole and ketoconazole significantly inhibited MDR3-mediated phospholipid secretion as well as BSEP-mediated bile salt secretion. Surprisingly, in parallel to MDR3 inhibition, posaconazole and itraconazole induced MDR3 protein levels by an unknown mechanism. In contrast, other hepatotoxic drugs such as co-amoxicillin, troglitazone and nefadozone did not affect MDR3 function suggesting that the hepatotoxicity of these compounds is not due to the inhibition of this transporter. Similarly, also the potential hepatoprotective compounds tested did not affect MDR3 activity in our model. In conclusion, the generated *in vitro* model to study bile salt and phospholipid secretion is functional and can be considered an inexpensive and reproducible system that facilitate

parallel screening for BSEP and MDR3 inhibitors, to study and predict drug-induced liver injury caused by inhibition of hepatobiliary transporters.

Sommario

La formazione di bile è un importante processo fisiologico finemente coordinato a livello della membrana canalicolare degli epatociti dall'azione di diverse proteine di transmembrana appartenenti alla superfamiglia dei trasportatori ABC (ATP-binding cassette). Tali proteine trasportano i costituenti della bile primaria nel canalicolo, uno spazio intercellulare delimitato, tra le membrane apicali di epatociti adiacenti. BSEP (bile salt export pump) pompa i sali biliari; mentre MDR3 (multidrug resistance protein 3) e ABCG5/G8 traslocano fosfatidilcolina rispettivamente colesterolo, rendendoli estraibili dai sali biliari nel canalicolo. Disfunzioni della secrezione biliare di sali biliari, fosfolipidi o colesterolo possono causare la colestasi o, in seguito alla cristallizzazione del colesterolo, la colelitiasi (calcoli biliari). Molti farmaci possono causare colestasi, alterando la funzione dei trasportatori canalicolari. Mentre la colestasi-farmaco indotta, dovuta a inibizione di BSEP, è un meccanismo ben studiato di colestasi intraepatica acquisita, studi concernenti interazioni di farmaci con MDR3 e sul ruolo dei trasportatori di lipidi come MDR3 nell'eziopatogenesi della colestasi farmaco indotta sono limitati. Lo scopo di questo progetto è stato di studiare l'interazione di vari farmaci in un modello cellulare polarizzato co-esprimente i principali attori della secrezione biliare. La prima parte del progetto si è focalizzata sulla caratterizzazione funzionale di un modello cellulare costituito da cellule LLC-PK₁ trasfettate stabilmente con NTCP, BSEP, MDR3 e ABCG5/G8 e polarizzate su filtri Transwell®. Analogamente alla situazione fisiologica nel canalicolo, il modello sviluppato esibiva sia trasporto vettoriale di sali biliari, che secrezione specifica di fosfatidilcolina nel compartimento apicale. In seguito, usando un analogo della fosfatidilcolina, C₆-NBD-PC, è stato sviluppato e validato un sistema per testare l'effetto di composti e farmaci sull'attività di MDR3. Per la seconda parte del progetto, una gamma di farmaci epatotossici o epatoprotettivi sono stati selezionati per valutare il loro effetto nel modello. Tra i diversi composti testati nel nostro modello cellulare gli azoli antifungini posaconazolo, itraconazolo e chetoconazolo inibivano significativamente sia la secrezione di fosfatidilcolina mediata da MDR3 che la secrezione di sali biliari mediata da BSEP. Inaspettatamente, l'effetto inibitorio di posaconazolo e itraconazolo sul trasporto di fosfatidilcolina era accompagnato da un'induzione dei livelli di proteici di MDR3 attraverso un meccanismo ancora sconosciuto. Invece, altri farmaci epatotossici, come co-amoxicillina, troglitazone e nefadozone non hanno avuto alcun effetto sul trasporto mediato da MDR3. Questo suggerisce che l'epatotossicità di questi composti non è dovuta ad un'inibizione di MDR3. Analogamente, anche i medicamenti potenzialmente epatoprotettivi testati, non hanno alterato la funzione di MDR3. In conclusione, il modello *in vitro* generato per studiare la secrezione

canalicolare di sali biliari e fosfolipidi è funzionale e può essere considerato un sistema rapido, economico e riproducibile per effettuare screening paralleli di inibitori di BSEP e MDR3 e quindi studiare e predire colestasi farmaco-indotta dovuta a un'inibizione dei trasportatori epatobiliari.

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List of abbreviations

ABC:	ATP-binding cassette
Alb:	albumin
ALT:	alanine aminotransferase
ALP:	alkaline phosphatase
AP:	apical
ATP:	adenosine triphosphate
BAS:	basolateral
BCRP:	breast cancer resistance protein
BN:	LLC-PK ₁ cell line transfected with BSEP and NTCP
BNMG:	LLC-PK ₁ cell line transfected with BSEP, NTCP, MDR3 and ABCG5/G8
BS:	bile salts
BSEP:	bile salt export pump
cat. #:	catalogue number
Caco-2:	human colorectal adenocarcinoma cell line
CMV:	cytomegalovirus
Co-amoxicillin:	amoxicillin and clavulanic acid
C ₆ -NBD-PC:	1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine
DILI:	drug-induced liver injury
DMEM:	Dulbecco's modified Eagle's medium
Fig.:	Figure
γ-GTP:	γ-glutamyltranspeptidase
HPTLC plate:	high performance thin-layer chromatography plate
MEM:	minimum essential medium
IC ₅₀ :	half maximal inhibitory concentration
LDH:	lactate dehydrogenase
LLC-PK ₁ :	pig kidney epithelial proximal tubule cell line
MDR:	multidrug resistance protein
MRP:	multidrug resistance-associated protein
NBD:	nucleotide-binding domain
NTCP:	Na ⁺ -taurocholate cotransporting polypeptide

OATP:	organic anion-transporting polypeptide
PBS:	phosphate-buffered saline
P450:	cytochromes P450 enzymes
PC:	phosphatidylcholine
PCR:	polymerase chain reaction
PFIC:	progressive familial intrahepatic cholestasis
PSC833:	Valspodar
SLC:	solute carrier
Tc:	taurocholate
TLC:	thin-layer chromatography
TBS-T:	tris-buffered saline/tween
TMD:	transmembrane domain
WT:	wild type

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1 Introduction

1.1 The liver and its role in bile formation and drug disposition

1.1.1 Overview

The liver is a vital organ and it represents, next to the skin, the largest organ in the body. In humans the liver is separated macroscopically into 4 lobes (Roth and Ganey, 2010). The principal cell type of the liver is the hepatocyte which accounts for 60% of the total cell population and 80% of the volume of the organ (Roth and Ganey, 2010). In addition there are endothelial cells that line the vascularizing vessels of the liver, stellate cells that store fat, Kupffer cells and lymphocytes that protect the liver. Generally the functions of the liver are carried out by the hepatocytes and include: 1) nutrient homeostasis (e.g. glucose and fatty acids synthesis); 2) protein synthesis (e.g. clotting factors and albumin); 3) biotransformation of endogenous (e.g. bilirubin, steroid hormones) or exogenous molecules (e.g. drugs and xenobiotics), also called detoxification and 4) bile formation (Klaassen, 2008).

1.1.2 Structural organization

Classically, the liver is divided into anatomical units called hepatic lobules (Klaassen and Watkins III, 2010). Hepatic lobules are hexagonal clusters of hepatocytes oriented around a hepatic central vein (Fig. 1). At the corner of the lobules there are the portal triads consisting of branches of a bile duct, portal vein, and hepatic artery (Klaassen and Watkins III, 2010). Venous blood from the stomach and the gut flows through the portal vein to the liver. In the liver, arterial and venous blood is mixed in the penetrating vessels, enters the sinusoids (small vascularizing vessels) and percolates along the cords of hepatocytes (Klaassen, 2008). Finally the blood exits the liver via the central vein. Along the portal-venous central-venous axis, hepatocytes display ultrastructural (e.g. mitochondria and smooth endoplasmic reticulum) and metabolic (various enzymes activities) heterogeneity (Jungermann, 1987; McCuskey, 2012). This results in a zonation of the hepatic lobule into regions with different cellular functions known as centrilobular, midzonal and periportal zones or acini (Klaassen, 2008; Roth and Ganey, 2010). For example biotransformation by oxidation reactions mediated by P450 enzymes are mainly centrilobular functions whereas glucose uptake occurs mainly in the periportal zone. Consequently also most pathological mechanisms have a certain degree of zonation.

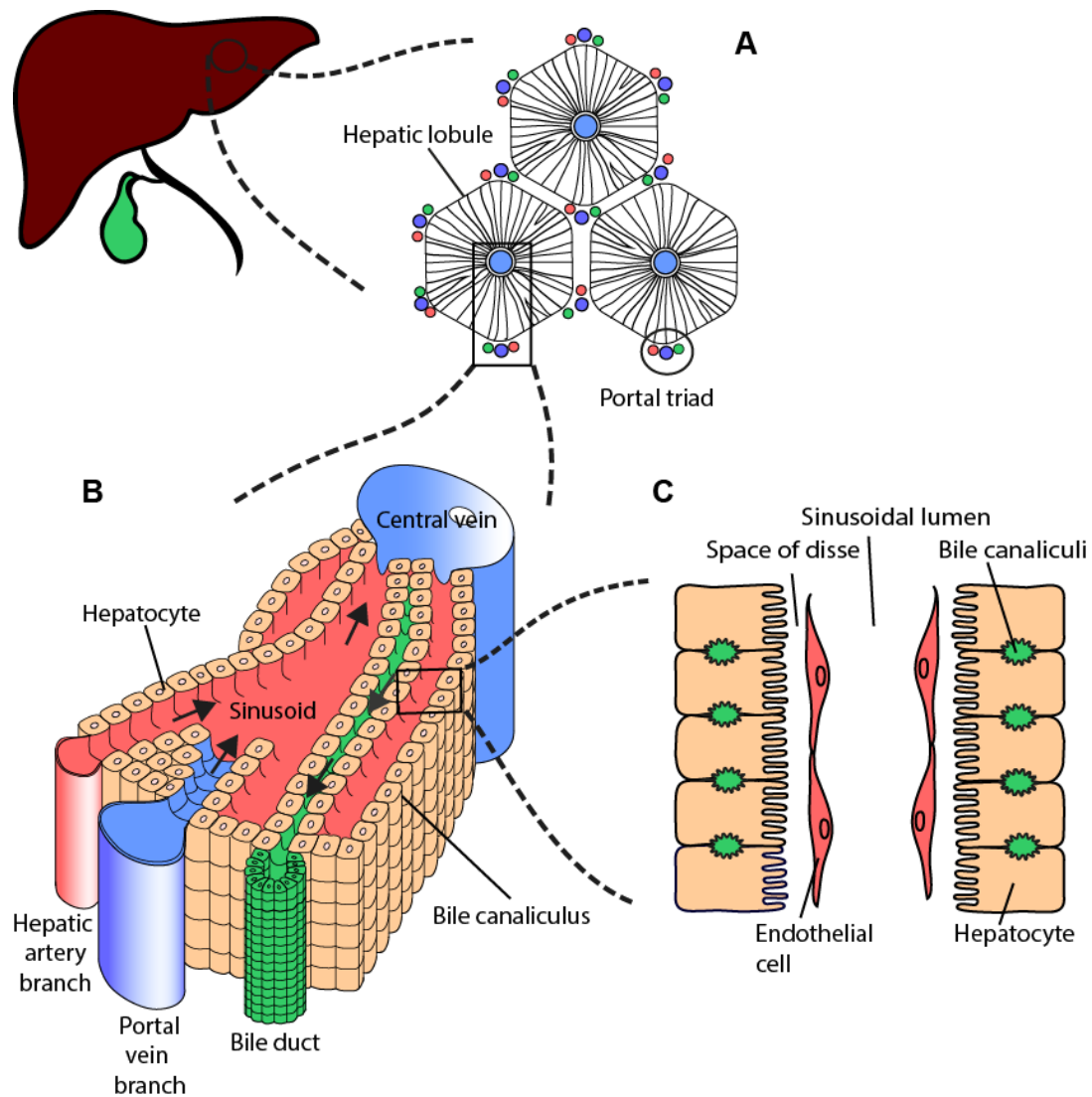


Fig. 1 The structural organization of the liver. (A) Schematic representation of the anatomical units of the liver called hepatic lobules. Hepatic lobules consist of hepatocytes, a central vein and 3 to 6 portal triads located at the corners of the structure. (B) 3D structure of a section of a hepatic lobule. Blood enters the liver from the portal veins and hepatic arteries and flows, countercurrent to the bile, into the central vein. (C) Architecture of the hepatic blood capillaries or sinusoids. Blood entering the liver from the portal veins and the hepatic arteries mixes in the sinusoids and flows through fenestrated endothelial cells to the space of disse, next to the hepatocytes. (B) and (C) were adapted by permission from Macmillan Publishers Ltd: *Nat Rev Immunol*, Adams and Eksteen, 2006, 6:244-51, copyright 2006.

1.1.3 Bile formation

One of the major functions of the liver is bile formation. Bile is a fluid constituted by bile salt (50% of dry weight), phosphatidylcholine (25% of dry weight), cholesterol, proteins, glutathione and small solutes and is important for the solubilisation and absorption of fats in the small intestine, for protection of the small intestine from oxidative insult and for the excretion of several endogenous and exogenous compounds (e.g. cholesterol, bilirubin and drugs) (Klaassen, 2008; Nicolaou et al., 2012). Hepatocytes represent the starting point of bile formation. They transport bile salts, glutathione, phosphatidylcholine and cholesterol into a space, between apical plasma membranes of adjacent hepatocytes, sealed by tight junction, and called canaliculus. From the canaliculus (~1 μm in diameter), primary bile, flows countercurrent to the direction of the portal blood flow, into the bile ducts and is finally delivered via larger bile ducts and the common bile duct into the gallbladder (Boyer, 2013).

At the molecular level the process of bile formation is coordinated by transport proteins expressed on the apical or canalicular membranes of hepatocytes (Fig. 2 and Fig. 4) belonging to the ABC transporter superfamily. Among the canalicular transporters, BSEP mediates the transport of conjugated bile salts from the hepatocytes into the canaliculus, the rate determining step of bile formation. MRP2 (multidrug resistance-associated protein 2) transports glutathione (GSH), as well as sulfated and glucuronidated bile acids and bilirubin diglucuronide (Klaassen, 2008; Pauli-Magnus and Meier, 2006). MDR3 and ABCG5/G8 translocate phosphatidylcholine and cholesterol, respectively, from the cytoplasmic to the outer leaflet of the canalicular membrane. The bile salts in the canaliculus facilitate the release of phosphatidylcholine from the outer leaflet of the canalicular membrane forming mixed bile salts-phosphatidylcholine micelles (Small, 2003). Formation of mixed bile salt-phosphatidylcholine micelles reduces the detergent activity of bile salts in the bile ducts (Trauner et al., 2008) and is crucial for the complete solubilization of cholesterol. Impairment in the process of bile secretion, as in the case of mutations of transporters such as BSEP, may result in the intracellular accumulation of toxic bile constituents e.g. bile salts in the hepatocytes, causing a reduced bile flow and hepatocellular damage, a condition called cholestasis (e.g. progressive familial intrahepatic cholestasis 2 or PFIC2) (Stieger, 2010). Beside the increase of intracellular levels of cytotoxic compounds, dysfunctions in the biliary cholesterol-bile salt-phospholipid secretion may lead to an alteration in bile composition. Alterations leading to an increased ratio of cholesterol to bile salt or phospholipids may result in cholesterol crystallization and finally in cholelithiasis, whereas alterations of bile composition resulting in a decreased ratio of phospholipids to bile salts may lead to “toxic bile” formation and subsequent hepatocellular and/or bile duct injury (Trauner et al., 2008).

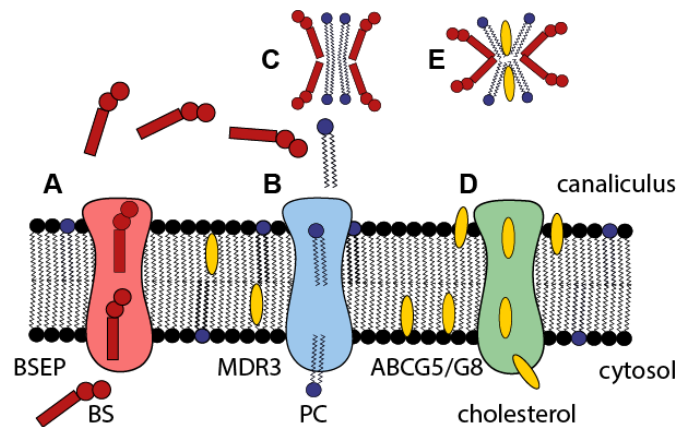


Fig. 2 Hypothesis of the molecular mechanisms underlying biliary lipid secretion. (A) BSEP pumps bile salts (BS) in the canalicular. (B) Bile salts in the canalicular pick up translocated phosphatidylcholine (PC) and (C) form PC-BS micelles. (D) PC-BS micelles incorporate cholesterol translocated by ABCG5/G8 and (E) form mixed BS-PC-cholesterol micelles which move down the biliary tract into the gallbladder and intestine. Adapted from *PNAS* (Small, 2003), Vol. 100, No. 1.

1.1.4 ABC transporters

ATP-binding cassette (ABC) transporters constitute the largest family of transmembrane transport proteins and are present in all three kingdoms of life (Ellinger et al., 2013; Locher, 2016). These proteins are primary active transporters moving a wide variety of substrates using ATP as energy source (Ellinger et al., 2013). Depending on the direction of the transport, if into the cytosol (inwardly) or out of the cytosol (out of the cell or into a cellular organelle), ABC transporters can be divided into two subtypes: importers and exporters (Holland et al., 2003; Hollenstein et al., 2007). ABC importers have been identified only in prokaryotes whereas ABC exporters are found in both eukaryotes and prokaryotes (Holland et al., 2003; Hollenstein et al., 2007). In addition, there is a third group of ABC transporters which does not seem to be directly involved in transport but rather in cellular processes such as DNA repair or regulation of gene expression (Holland et al., 2003). All ABC transporters share a basic structure consisting of two hydrophilic cytoplasmic nucleotide-binding domains (NBDs), that bind and hydrolyze ATP, and two hydrophobic transmembrane domains (TMDs) that form a translocation pathway for substrates across the membrane bilayer (Fig. 3). These four domains can be encoded as four independent polypeptides (mostly in bacterial importers), as one single polypeptide (also called full transporters, mostly in eukaryotic exporters) or as two multidomain polypeptides (halftransporters). The NBDs of all ABC transporters share extensive amino acid sequence identity and several characteristic motifs such as P-loop and the LSSGQ motif (Higgins and Linton, 2004; Locher, 2009). In contrast, the TMDs, which consist of multiple membrane spanning α -helices arranged with an internal pseudo two-fold symmetry, can contain different numbers of helices (6 to 11) and share little amino acid sequence identity (Higgins and Linton, 2004). This may also explain the specific characteristics of ABC transporters as the large

diversity of the transported substrates. Although many details are not known yet, a common general mechanism of transport has been proposed. According to the current models, the “ATP-switch model” and the “alternating-access model”, dimerization of the NBDs, upon binding of ATP, translates into a conformational change of the TMDs. Alternation of the TMDs between inward and outward facing conformation promotes transport. The direction of transport is determined by the different binding affinity of the substrate for the TMDs conformations. However, recent data also support that ABC-mediated transport can occur without an inward-facing opening of the transporter (Perez et al., 2015).

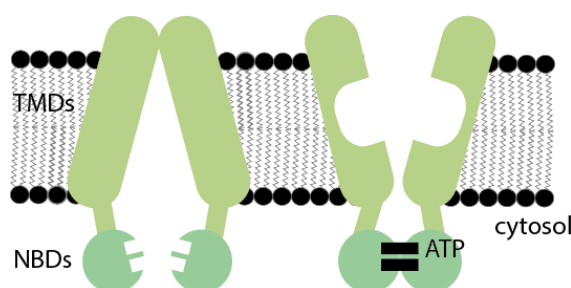


Fig. 3 Models of the open (inward facing) and closed conformations (outward facing) of ABC transporters. Dimerization of the NBDs upon binding of ATP is transmitted by coupling helices to the TMDs which change conformations. Adapted from *Microbiology and Molecular Biology Reviews* (Cuthbertson et al., 2010), Vol. 74, No. 3, Copyright 2010, American Society of Microbiology.

ABC transporter function can be altered by modulators/inhibitors. Most knowledge on direct modulators of ABC protein derives from the drug interaction studies performed on ABCB1 which is the multidrug-resistance protein 1 (MDR1, and most commonly known as P-glycoprotein). From these studies, two general modes of inhibition of ABC transporters emerged: 1) Some inhibitors (e.g. verapamil) may bind and compete for the same or overlapping binding sites of the substrates, in the TMDs. In this case transport of a substrate is inhibited in a competitive manner; 2) Some modulators may bind to the NBDs (e.g. flavonoids) and suppress the ATPase activity. In this case, the transport of a substrate is inhibited in a non-competitive manner.

The human genome encode 48 genes for ABC transporters, most of which are thought to be exporters. In humans, ABC proteins are either full transporters or dimers of half transporters. Based on sequence similarity and the organization of the domains, the Human Genome Organization have grouped these proteins into 7 families, from ABCA to ABCG (Dean, 2002). Since ABC transporters defects have been implicated in several monogenetic diseases, in increased susceptibility to specific complex disorders (Dean, 2002) and as cause of multidrug resistance (Wu et al., 2008) (Table 1), a better molecular understanding of the function and structure of these proteins is of clinical importance and may help in the development of specific modulators with potential beneficial medical effects or in the design of safer drugs.

Table 1 Classifications of ABC transporters and example of diseases associated with their loss of functions mutations. Adapted from Dean (Dean, 2002).

Family	Example	Disease (loss of function)
ABCA		
12 full transporters	ABCA1	Tangier disease
ABCB		
	ABCB11/BSEP	PFIC2
4 full transporters and 7 half transporters	ABCB4/MDR3	PFIC3
ABCC		
	ABCC7/CFTR	Cystic fibrosis
	ABCC2/MRP2	Dubin-Johnson syndrome
12 full transporters	ABCC6	Pseudoxanthoma elasticum
ABCD		
4 half transporters, all in peroxisomes	ABCD1/ALD	Adrenolekodystrophy
ABCE and ABCF		
	ABCE	
1 ABCE and 3 ABCF, no TMD only NBD	ABCF1	
ABCG		
6 half transporters	ABCG5/G8	Sitosterolemia

Of the 48 members of the ABC superfamily that have been identified in humans, 14 have been detected at the protein level in the liver (Wlcek and Stieger, 2014) and play a key role in bile formation (see previous section). In the liver, mutations in the *ABCB11* and in the *ABCB4* genes encoding BSEP and MDR3, respectively, have been linked to various hepatobiliary disorders such as progressive familial intrahepatic cholestasis or susceptibility to intrahepatic cholestasis of pregnancy (Trauner et al., 2007). Mutations of BSEP are characterized by increased intracellular levels of bile salts and a decreased bile flow. The pathophysiological mechanisms underlying hepatocellular and bile duct injury in patients with mutations of MDR3 are not fully understood but it has been suggested that impairment of MDR3 may lead to the formation of “toxic bile” (Trauner et al., 2008). Consistent with the concept of “toxic bile”, MDR3 knockout mice models develop bile duct injury with features resembling those observed in human sclerosing cholangitis suggesting that formation of toxic bile could also play an important role in the pathogenesis of various cholangiopathies in humans (Trauner et al., 2008). Mutations in *ABCC2* encoding MRP2 results in the Dubin-Johnson syndrome, a disease characterized by conjugated hyperbilirubinemia. *ABCG8* gene polymorphisms (p. D19H and p. T400K) lead to increased cholesterol secretion into the bile and were linked to an increased risk of gallstone disease in certain populations (Rudkowska and Jones, 2008). MDR1 or P-glycoprotein, discovered as conferrer of multidrug resistance in tumor cells (Huang et al., 1998), mediates export of

drugs and hydrophobic substrates from the cells (International Transporter et al., 2010). The impact of MDR1 polymorphisms on the liver is not clear; however it is conceivable that by increasing drug, xenobiotic and metabolite concentration in the hepatocytes, it may lead to an increased risk for drug-induced injury (Pauli-Magnus et al., 2010).

1.1.5 Hepatic drug disposition

Another major function of the liver is detoxification. The liver is a primary organ involved the disposition of drugs, xenobiotic as well as endobiotic substances. After oral administration, xenobiotics and drugs are absorbed across the intestinal wall and are delivered to the liver by the portal hepatic system (Niemi, 2007). In the liver, xenobiotics and drugs flowing in the sinusoids cross the interstitial space and are taken up mainly by carriers expressed at the basolateral membrane of hepatocytes (Shugarts and Benet, 2009). In hepatocytes, endobiotics and xenobiotics are eventually bio-transformed into water-soluble metabolites by Phase I and Phase II enzymes before being finally secreted by efflux pumps either back into the blood through the basolateral membrane, or into in the bile, through the canalicular membrane of hepatocytes. Classical drug disposition has been considering only Phase I and Phase II reactions (Szakacs et al., 2008). However, in the last decades, with the increasing recognition of the important role of transporters in drug uptake and elimination and thus as determinants of drug pharmacokinetic and pharmacodynamics, the concept of drug disposition has been extended to drug uptake or Phase 0 and drug efflux or Phase III (Vavricka et al., 2002).

1.1.5.1 Hepatic uptake (Phase 0)

Hepatic uptake mechanisms determine how exogenous and endogenous solutes enter (Phase 0) the hepatocytes. Phase 0 uptake is mediated by transporters belonging mainly to the SLCs superfamily (Shugarts and Benet, 2009). The SLC superfamily members encompass a variety of transporters that use different transport mechanisms (Shugarts and Benet, 2009). They can be passive (uniporters or facilitative transporter) or secondary active transporters. Passive transporters move substrates down their electrochemical gradient. In contrast, secondary active transporters move substrates against their electrochemical gradient by coupling with another molecule that flows downhill its concentration gradient. Secondary active transporters can be coupled/symporters or exchangers/antiporters (Dobson and Kell, 2008). In the liver, the sodium-taurocholate co-transporting polypeptide (*SLC10A1*, NTCP) and the organic anion transporting polypeptides (SLCO or *SLC21A*, OATPs) are SLCs transporters expressed on the basolateral membrane of hepatocytes and play the predominant role in the uptake of bile salts and drugs. NTCP is a secondary active, sodium-coupled transporter that mediates uptake of conjugated bile salts into the hepatocytes and hence it is fundamental for the maintenance of the enterohepatic cycle of bile salts. Its energy is derived from the sodium gradient which is maintained by the ATP-dependent Na^+/K^+ -ATPase which is also localized at the basolateral membrane (Boyer,

2013). Currently, the role of this transporter in drug and xenobiotic uptake seems to be limited, as the only known substrate is the HMG-CoA reductase inhibitor rosuvastatin (Ho et al., 2006). However, a genetic polymorphism of NTCP found in asiatic population showed a gain for rosuvastatin transport activity (Ho et al., 2006) suggesting that in certain cases NTCP could play a more important role in drug disposition. Furthermore, various drugs have been reported to inhibit NTCP e.g. cyclosporin (Stieger, 2011) and therefore NTCP may be relevant in drug interactions. In contrast, the mechanism of substrate uptake by OATPs transporters is not completely understood but they are believed to act as organic anions exchangers (Hagenbuch and Stieger, 2013), in which the cellular uptake of organic anions is coupled to the efflux of neutralizing anions such as bicarbonate, glutathione or glutathione-*S*-conjugates. However, the nature of the neutralizing ion remains unknown for human OATPs (Niemi, 2007). Generally, OATPs have been detected in essentially every organ in epithelial or endothelial cells. Some OATPs have a restricted expression and are therefore assumed to be organ specific, while others are expressed ubiquitously (Hagenbuch and Stieger, 2013). OATP1B1 and OATP1B3 are considered to be the liver-specific OATPs (Hagenbuch and Stieger, 2013; Obaidat et al., 2012). OATP1B1 is expressed in hepatocytes throughout the lobule, while OATP1B3 is primarily expressed around the central vein (Hagenbuch and Stieger, 2013). In contrast to NTCP, OATP1B1 and OATP1B3 contribute to a lesser extent to the uptake the bile salts, but play a major role in transporting endobiotics, xenobiotics and drugs into the hepatocytes. Drug substrates of OATP1B1 include a large number of structurally diverse compounds, such as statins (atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin acid), the endothelin receptor antagonists atrasentan and bosentan, the antibiotics benzylpenicillin and rifampicin, the antifungal agent caspofungin, the angiotensin-converting enzyme inhibitors enalapril and temocapril, the angiotensin II receptor antagonists olmesartan and valsartan and many others (Niemi, 2007). OATP1B3 transports several therapeutic drugs, with overlapping substrate specificity with OATP1B1 (Niemi, 2007). However, OATP1B3 is unique among hepatic OATPs in transporting digoxin, docetaxel and paclitaxel (Niemi, 2007).

1.1.5.2 Biotransformation reactions (Phase I and II)

Biotransformation is the chemical conversion of endogenous or exogenous compounds into more water-soluble compounds that can be more easily excreted by the body (Klaassen and Watkins III, 2010). Phase I biotransformation reactions of drugs and xenobiotics are carried out primarily by cytochrome P450 enzymes and involve hydrolysis, reduction and oxidation reactions. These reactions occur in the endoplasmic reticulum and usually expose or introduce a functional group (-OH, -NH₂, -SH or COOH) resulting in a small increase of hydrophilicity (Klaassen and Watkins III, 2010) and most importantly producing a reactive site on the molecule suitable for conjugation reactions (Phase II). As a consequence, these reactions can have both, protective or toxic effects as they may inactivate

toxic chemicals to innocuous compounds or may also activate drugs to toxicants. By developing “prodrugs” (inactive drugs) that are intracellularly activated to the pharmacologically active agent, the pharmaceutical industry has exploited Phase I reactions to improve drug pharmacokinetics and decrease drug toxicity. Phase II biotransformation reactions occur predominantly in the cytosol but also in the endoplasmic reticulum and include glucuronidation, sulfonation, acetylation, methylation, conjugation with glutathione and conjugation with amino acids (Klaassen and Watkins III, 2010). The substrates react with functional groups which are either present on the xenobiotic or introduced during Phase I reactions. Generally, these reactions result in a large increase in hydrophilicity enabling these substances to be secreted during Phase III.

1.1.5.3 P450s enzymes

The human genome contains 115 CYP genes, of which 57 are functional cytochrome P450 (CYP) genes (Johansson and Ingelman-Sundberg, 2011; Zanger and Schwab, 2013) encoding heme-containing enzymes. Based on sequence similarity (Kaplowitz and DeLeve, 2003) CYPs are divided into families indicated by a number. CYP families can be categorized into 2 major groups: 1) CYP families 4-51 are mainly involved in the biosynthesis and metabolism of endogenous compounds (e.g. sterols, eicosanoids, fatty acids). They usually have high affinity for the substrates and are well conserved during evolution; 2) CYP families 1-3 are involved in the metabolism of exogenous compounds as drugs and xenobiotics. They have usually less affinity for their substrates, are less conserved evolutionary and exhibit important genetic polymorphisms (Ingelman-Sundberg, 2004; Johansson and Ingelman-Sundberg, 2011; Kaplowitz and DeLeve, 2003).

The second group (CYP families 1-3) is responsible for 75% of all Phase I dependent metabolism of clinically used drugs (Johansson and Ingelman-Sundberg, 2011). Within this group, 6 isozymes have been identified to play a predominant role in drug metabolism: CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP2E1, and CYP3A4. Each isoenzyme can metabolize several drugs and many drugs can be metabolized by more than one CYP/P450. This may lead to drug-drug interactions, when combinations of 2 or more drugs being metabolized by the same isoenzyme are co-administered (as metabolism of a drug would be competitively inhibited by the other). In addition, induction (increased enzyme expression) or inhibition of P450 by drugs or xenobiotics can accelerate or slow drug metabolism leading to a decreased therapeutic efficacy of a drug or drug-induced toxicities, respectively. For example cyclosporin and simvastatin are substrates of CYP3A4. Co-administration of these drugs with CYP3A4 inhibitors such as ketoconazole or grapefruit juice decreases cyclosporin and simvastatin dosage requirement (Ogu and Maxa, 2000). This is not a problem if serum levels of the drugs would be monitored and dosage would be adjusted. However, since for statins serum levels are usually not measured, co-administration of a CYP3A4 inhibitor may increase the risk for toxic drug effects such as rhabdomyolysis. On the other hand co-administration of cyclosporin and

simvastatin drugs with CYP450 inducers such as St. John's wort or rifampicin may lead to increased elimination of these drugs and subtherapeutic drug concentrations. For cyclosporin, this potentially leads to transplant rejection (Barone et al., 2000). Finally, beside drug interactions, also genetic polymorphisms affecting P450 may also alter P450 enzymes activities and therefore are very important to understand interindividual variability in drug response (Fujikura et al., 2015).

1.1.5.4 Hepatic efflux (Phase III)

The efflux of endogenous or exogenous compounds, including drugs and their metabolites, from the hepatocytes represents the last step of the hepatic disposition of drugs and is called Phase III. Phase III is mediated by ABC transporters (Doring and Petzinger, 2014). ABC transporters are primary active transporters that export compounds against their concentration gradient. Most ABC transporters in the liver localize on the apical membrane of hepatocytes (Fig. 4) and their main physiological function is the secretion of bile constituents for primary bile formation in the canaliculus. In the canaliculus, one of the major determinants of drug disposition is MDR1 (Pauli-Magnus and Meier, 2006). MRP2 mediates the transport of glucuronidated compounds into the bile (e.g. bilirubin and drugs), whereas BCRP display a preference for sulfated conjugates (de Lima Toccafondo Vieira and Tagliati, 2014). In addition, ABC transporters belonging to the family of multidrug resistance-associated proteins (MRPs) are expressed on the basolateral membrane. These move substrates back into the disse space (interstitial space) and thus into the blood. MRP3 and MRP4 have been shown to play a protective role by mediating basolateral bile salts efflux when intracellular levels of bile salts rise and are involved in the transport of the antiretroviral drugs lamivudine, zidovudine and stavudine (de Lima Toccafondo Vieira and Tagliati, 2014).

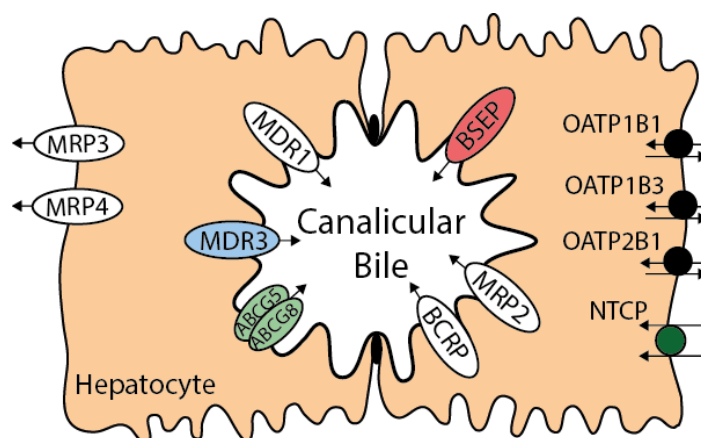


Fig. 4 Bile and drugs transporters in human liver hepatocytes. SLCs transporters: round symbols; ABC transporters: ovals symbols. Shown in colors (blue, green and red) are the key transporters involved in bile formation.

1.2 Drug-induced liver injury

1.2.1 Overview

As the first and main organ responsible for the uptake (Phase 0), metabolism (Phase I and II) and excretion (Phase III) of the majority of drugs, xenobiotics and their metabolites as well as endogenous compounds, the liver is particularly predisposed to drug toxicity. Indeed, factors affecting these processes may increase the level of exposure of hepatocytes to exogenous molecules such as drugs and/or their metabolites or alternatively, alter disposition of endogenous molecules, contributing to the development of drug-induced liver injury (DILI). Although, because of underreporting and an unknown denominator of persons assuming a drug, the true incidence of DILI is very difficult to assess, about 10-20 cases per 100'000 persons have been estimated to occur each year (Bjornsson et al., 2013; Leise et al., 2014). DILI represents an important clinical problem for the following major reasons: 1) it accounts for more than 40% of hepatitis in adults (Pauli-Magnus and Meier, 2006) and for most of the cases of acute liver failure both in the USA and Europe (Ramachandran and Kakar, 2009; Reuben et al., 2010; Wei et al., 2007); 2) it is a primary cause for withdrawal or labeling changes of drugs after their approval, and is therefore a critical issue for the pharmaceutical industry and for the drug regulatory agencies (Cheng et al., 2011; Corsini and Bortolini, 2013; Yang et al., 2015), since the potential toxicity of many drugs is not evident during clinical phases (Kaplowitz and DeLeve, 2003); 3) it encompasses a broad spectrum of liver injuries and can mimic all forms of liver diseases and therefore represents a diagnostic challenge for physicians (Kaplowitz and DeLeve, 2003). DILI diseases can be nonidiosyncratic/predictable (high incidence and dose related e.g. paracetamol overdose) or idiosyncratic/unpredictable (low incidence and only above a threshold dose-related e.g. amoxicillin-clavulanate) (Chughlay et al., 2015; Leise et al., 2014). Generally, early manifestations of toxicity, within a few days from starting drug exposure, is a characteristic of predictable reactions, whereas unpredictable reactions usually occur with intermediate (1-8 weeks) or long latency (up to 12 months) (Kaplowitz and DeLeve, 2003).

DILI is typically classified biochemically into three patterns of injury on the basis of the relative changes of the serum enzymes: a hepatocellular pattern is characterized by a predominant increase in alanine aminotransferase (ALT), a cholestatic pattern by a predominant increase of alkaline phosphatase (ALP), and a mixed hepatocellular-cholestatic pattern by a moderate increase of ALT and ALP where the latter is the most characteristic of DILI (Hussaini and Farrington, 2007; LiverTox.nih.gov[2016]). The R ratio has been defined to assess the pattern of DILI (Table 1) (Hussaini and Farrington, 2014).

Table 2 Classification of drug-induced liver injury according to CIOMS (Council for International Organizations of Medical Sciences). ALP: alkaline phosphatase; ALT: alanine aminotransferase; ULN: upper limit of normal. Table adapted from Hussaini et. al. 2014.

Liver injury pattern	$R = (ALT/ULN) / (ALP/ULN)$
Hepatocellular	$R \geq 5$
Mixed	$R > 2$ and < 5
Cholestatic	$R \leq 2$

If the biochemical changes are present since more than 3 months, a liver injury pattern is considered chronic (Ramachandran and Kakar, 2009). In addition, several clinicopathological classifications of DILI have been proposed (Lewis, 2000; Ramachandran and Kakar, 2009; Zimmerman, 2000). Considering clinical features, laboratory and histological findings, clinicopathological classifications categorize DILI into more specific categories or phenotypes (Farrell, 1994; LiverTox.nih.gov[2016]). According to this approach DILI is categorized into: acute or chronic hepatitis, steatosis, phospholipidosis or nonalcoholic fatty liver (all hepatocellular injuries), bland cholestasis, cholestatic hepatitis, vascular disorders (sinusoidal dilatation, sinusoidal obstruction, peliosis hepatitis etc.) and hepatic tumors (such as hepatic adenoma and hepatocellular carcinoma) (Farrell, 1994; LiverTox.nih.gov[2016]). However, the limitation of this classification is that many drugs can produce more than one type of clinicopathological syndrome (Farrell, 1994).

1.2.2 Pathogenesis of DILI

The pathogenesis of DILI is complex and not fully understood (Chughlay et al., 2015). Several cellular mechanisms with multiple variable outcomes have been involved in the pathogenesis of DILI (Fontana, 2014). One of the key challenges associated with DILI is why some drugs are well tolerated by the majority of the patients while some patients are susceptible and develop liver injury (Roth and Ganey, 2010). The prevailing opinion is that enhanced genetic susceptibility (e.g. polymorphisms of P450 enzymes or transporters) and/or failure to adapt to a cellular drug response are key factors leading to DILI (Klaassen, 2008). From the mechanistic point, although interconnected between each other, four pathways of toxicity underlying the pathogenesis of DILI can be differentiated: 1) oxidant/electrophilic stress; 2) immune mechanisms; 3) mitochondrial toxicity; 4) inhibition of hepatic transporters (Roth and Ganey, 2010).

1.2.2.1 Oxidant and electrophilic stress

Metabolism of drugs by Phase I enzymes produces metabolites that are generally safely eliminated from the body by conjugation reactions catalyzed by Phase II enzymes. However, under certain circumstances such as genetic polymorphisms of Phase I (P450) or Phase II enzymes, induction of

certain Phase I enzymes, or low expression of Phase II enzymes, reactive and potentially toxic metabolites are generated (Kaplowitz and DeLeve, 2003) and may accumulate in the hepatocytes. Briefly, a balance between Phase I and Phase II reactions determines whether the reactive metabolites are safely detoxified (Klaassen, 2008). These reactive intermediates are electrophilic metabolites that covalently bind to nucleophilic residues on proteins generating a complex (hapten) that may induce an immune response (Fontana, 2014; Kaplowitz, 2004; Roth and Ganey, 2010). Alternatively, the reactive metabolite may directly damage the cell by glutathione depletion, covalent binding to cellular constituents such as protein, lipids or DNA or inducing lipid peroxidation (Kaplowitz, 2004).

1.2.2.2 Immune mechanisms

Innate and adaptive immune mechanisms may trigger DILI or may be activated by DILI triggered by other mechanisms such as electrophilic stress or cholestasis. The observation of rash, fever, eosinophilia after 1-8 week of drug treatment and the detection of antibodies against hepatic proteins support the involvement of an adaptive immune response (Ju and Reilly, 2012). The main hypothesis is that the drug or its metabolites covalently bind to a liver protein such as cytochrome P450 and act as hapten (Yuan and Kaplowitz, 2013). Presentation of the processed drug-protein adducts by antigen presenting cells triggers the adaptive immune response. Responses of the adaptive immunity can be humoral or cellular (Roth and Ganey, 2010). Humoral responses are mediated by B-cells and involve the generation of antibodies against the hapten or autoantibodies against physiological proteins such as P450 enzymes. Alternatively, cellular responses may lead to CD8 cytotoxic T-cell activation (Yuan and Kaplowitz, 2013). CD8 T cells express FasL, TNF- α , and perforin that mediate cell death of hepatocytes. In addition, necrosis of hepatocytes leads to the activation of innate immune cells to produce cytokines such as TNF- α , IFN- γ , and IL-1 (Yuan and Kaplowitz, 2013). This results in inflammation (hepatitis). Hence, although the liver is known as a site of immune tolerance (Crispe 2014), the immune system can play an important role in different mechanisms of DILI.

1.2.2.3 Mitochondrial toxicity

Mitochondria are the most abundant organelles in the liver (Kaplowitz and DeLeve, 2013). The main function of hepatic mitochondria is oxidative phosphorylation to generate ATP. For this, pyruvate produced by glycolysis, acyl-CoA resulting from beta-oxidation of fatty acids and amino acids obtained from the protein catabolism are oxidized through the citric acid cycle to yield NADH and FADH₂. The flow of electrons from NADH, through protein complexes in the mitochondrial membrane, generates a proton gradient that leads to the synthesis of ATP during oxidative phosphorylation. Mechanisms impairing mitochondrial function include inhibition of beta oxidation of fatty acids (e.g. amiodarone, valproate) leading to fatty liver (steatosis) (Amacher and Chalasani, 2014), inhibition of oxidative phosphorylation (e.g. by NSAIDs such as nimesulide), depletion of mitochondrial DNA and promotion of permeabilization of the inner mitochondrial membrane (e.g.

troglitazone) (Labbe et al., 2008; Masubuchi et al., 2006). The impaired ability of mitochondria to generate ATP could be implicated in the functional suppression of ABC-transporters during cholestasis (Masubuchi et al., 2006).

1.2.2.4 Interactions with/Inhibition of hepatic transporters

Bile formation is a fundamental function of the liver. Failure of bile secretion or flow due to an obstruction of the bile ducts or an impairment of uptake or efflux processes mediated by the hepatocytes is a pathophysiologic process named cholestasis (Jaeschke et al., 2002; Roth and Ganey, 2010). Direct or indirect inhibition of hepatobiliary transporter proteins by parent drugs or metabolites underlie these reactions (Pauli-Magnus and Meier, 2006). Certain drugs may either directly inhibit transport proteins by non-competitive or by competitive (substrates of transport proteins) interactions. Alternatively, hepatobiliary transporters may be indirectly inhibited for example by downregulation of their expression. Retention of bile constituents such as bile salts within the hepatocytes by inhibition of BSEP by drugs is an example for such a reaction. Cyclosporin A, rifampicin, bosentan and glibenclamide are examples of drugs that are competitive inhibitors of human BSEP and that lead to increased intracellular levels of bile salts within the hepatocytes (Stieger, 2010). Elevated concentrations of bile salts trigger translocation of intracellular Fas bearing vesicles to the plasma membrane where they self-aggregate (Jaeschke et al., 2002). Activated Fas receptors complex on the plasma membrane causing caspase 8 activation and thus apoptosis (Jaeschke et al., 2002). Alternatively, bile salts may activate pro-apoptotic kinases (PKC- δ and JNK1/2) (Jaeschke et al., 2002). Consistent with the important role of BSEP in drug-induced cholestasis, most drugs implicated in cholestatic injury are reported to inhibit BSEP and the list of BSEP inhibitors is continuously growing (de Lima Toccafondo Vieira and Tagliati, 2014). Moreover, several variant mutations of BSEP have been identified, of which the V444A polymorphism is particularly associated with drug-induced cholestasis (Bhamidimarri and Schiff, 2013). Finally, considering that MDR3 variants are also a susceptibility factor for DILI, that recently MRP4 inhibition has also been associated to DILI (Kock et al., 2014) and that prediction of DILI has been reported to be improved by considering MRP2, MRP3 and MRP4 as compared to BSEP alone (Morgan et al., 2013) it seems reasonable to believe that the role of transporters in the development of DILI is even more important than what it has been thought.

1.2.3 Tools for the identification of drugs with cholestatic potential

A better understanding and prediction of drug transporter interactions is crucial to design and develop safer drugs. Therefore, several *in vitro* and *in vivo* models have been developed to assess inhibition of transporters by drugs. *In vivo* models are mainly used in drug development to ensure that drugs which enter clinical trials do not cause dose-dependent liver injury in humans, but they are of limited value for the clarification of the mechanisms underlying rare idiosyncratic DILI (Greer et al., 2010). This is due to several reasons, including: 1) their complexity: generally, if several mechanisms or transporters are involved in the development of the liver injury it can be difficult to assess the individual contribution of each one in an organism model. 2) Compensatory mechanisms can be activated: for example, in genetically modified animals, loss of function of one transporter may result in compensatory changes in other pathways (Yang et al., 2016). 3) Finally, there can be important differences between human and animal homolog proteins: for example, expression levels, or characteristics such as affinity to a drug or substrate can be very different. Therefore, *in vitro* assays have been established to investigate drug transporter interactions. *In vitro* models are fast, reproducible (de Lima Toccafondo Vieira and Tagliati, 2014), are well controlled and simplified model systems, and can be used also in high-throughput screenings. The types of *in vitro* models that have been developed include vesicle-based and cell-based models. Membrane vesicles are usually prepared from organs such as the liver, which naturally expresses the transporters, or from transfected cell lines such as baculovirus-infected insect cells or mammalian cells (Zhang et al., 2012). Vesicle-based assays can be used for vesicular transport/uptake assays, where the amount of transported labelled substrate is quantified, or for ATPase assays, where the amount of hydrolyzed ATP in the presence of inhibitors or substrates is determined (de Lima Toccafondo Vieira and Tagliati, 2014). ATPase assays indirectly give information on the nature of the interaction occurring (if inhibitory or stimulatory) and their use is limited to ATP-dependent transporters. Vesicular transport assays can be used to measure the transport mediated by both SLC and ABC transporters using labelled substrates. For example, functional assessment of BSEP inhibition by drugs is commonly performed using isolated membrane vesicles obtained from cells overexpressing BSEP (Stieger, 2010). This approach is now widely used to either explain cholestatic events in retrospective (de Lima Toccafondo Vieira and Tagliati, 2014) or to investigate the cholestatic potential of new chemical entities during drug development (Thompson et al., 2012). Cell-based models include hepatocytes, sandwich cultured hepatocytes and transfected cell models. Hepatocytes-based models can be especially useful if contribution of P450-mediated drug metabolism in the development of DILI should be assessed (Yang et al., 2016; Zhang et al., 2012). On the other hand, transfected cell models can be very efficient to characterize drug interactions with a transporter, as they generally display a higher signal-to-noise ratio for the studied transporter. Despite the efficiency of this model to characterize drug interactions with a transporter and to make qualitative predictions, one should keep in mind that the

levels of the transfected proteins may be very different from the *in vivo* situation. Therefore, extrapolation from *in vitro* to *in vivo* should be carefully made. Transfected cell models can be both non-polarized, e.g. HEK 293 (human embryonic kidney cells) and CHO cells (chinese hamster ovary cells), or polarized as in the case of Caco-2, LLC-PK₁ (pig kidney proximal tubule cells) and MDCK (Madin-Darby canine kidney cells). An additional cell-based model is represented by *Xenopus laevis* oocytes. Non-polarized cell models can be used for uptake assays, where at the end of the assays cells are lysed and intracellular accumulation of a substrate is determined. Polarized cell models can be cultured as tight monolayers in the Transwell[®] system to develop a two-compartment system and therefore can be used to study transport across monolayers (e.g. mimicking intestinal absorption with Caco-2 cells). The Transwell[®] system provides accessibility to both basolateral and apical domains allowing drug, substrate and acceptor supplementation to both or only one compartment. However, when polarized cell models are used to study efflux processes, they may require co-expression of uptake transporters (Zhang et al., 2012). Cell-based models have the advantage over vesicle-based models that the cells express other enzymes and co-factors that could be important for the function of the studied transporter. However, as compared to vesicle-based models, cell-based models can be more susceptible to cytotoxic effects of drugs (therefore it is not always possible to determine IC₅₀ values). In summary, each of the *in vitro* methods presents its advantages and challenges in predicting the cholestatic potential of drugs (de Lima Toccafondo Vieira and Tagliati, 2014) and it is very likely that only a combination of different *in vitro* methods with *in silico* approaches and clinical available data will improve predictability and understanding of complex diseases such as DILI.

2 Aims of the work

As outlined in the previous section, *in vitro* model systems are useful tools to predict and understand the mechanisms underlying drug hepatotoxicity. Several tools have been already established for SLCs and ABC transporters as OATPs, NTCP, MDR1, BSEP and MRP2. However, for biliary lipid transporters, very little has been done. Considering the physiological role of MDR3 in reducing the toxicity of bile in the bile ducts, and the effect of inherited mutation of MDR3 in patients it is conceivable that also interactions with MDR3 may play a role in the mechanism underlying DILI. Therefore, we aimed to: 1) Establish a polarized, *in vitro* model for canalicular lipid secretion, expressing human MDR3, BSEP, ABCG5/G8 and NTCP, and displaying vectorial bile salt and lipid transport in Transwells. Using Transwells we wanted to ensure direct experimental access to both basolateral as well as apical compartments which allows testing different conditions as well as preloading of the apical compartment with acceptor proteins to ensure solubility of the translocated lipids into the apical medium; 2) Develop and validate sensitive and practical assays to measure phospholipid and cholesterol secretion in the established model. To date MDR3-mediated phospholipid secretion has been often studied using phospholipid precursors such as radioactively labelled choline (Yoshikado et al., 2011) or fluorescent labelled diacylglycerol (van Helvoort et al., 1996). However these approaches are susceptible to alterations of lipid biosynthesis. 3) Investigate the impact of drugs/compounds on bile salt, phospholipid secretion and clarify whether inhibition of MDR3 may be a potential mechanism contributing to the toxicity of drugs reported to induce cholestatic DILI 4) Search *in vitro* for stimulators of canalicular lipid secretion that may have beneficial effects on injured bile ducts *in vivo*.

3 Materials and methods

3.1 Materials

3.1.1 Eukaryotic cell lines

- **LLC-PK₁**, pig kidney epithelial proximal tubule cell line (ATCC, Manassas, VA, USA).
- **Caco-2**, human colorectal adenocarcinoma cell line (ATCC, Manassas, VA, USA).

3.1.2 Substrates for transport assays

Table 3

Substrates	Company and cat. #	Conc. used
¹⁴ C-cholesterol	PerkinElmer, NEC018	1 μM, 0.5 μCi/ml
C ₆ -NBD-PC	Avanti Polar Lipids, 810130C	12.5 μM
³ H-taurocholic acid	PerkinElmer, NET322	0.429 μM, 0.2 μCi/ml
Taurocholic acid	Sigma, T4009	9.957 μM

3.1.3 Buffers and solutions for Western blot

- **Electrode buffer:** 192 mM glycine, 25 mM Trizma[®] base, 0.1% (w/v) SDS, dH₂O
- **Transfer buffer:** 192 mM glycine, 25 mM Trizma[®] base, 0.1% (w/v) SDS, 20% (v/v) methanol, dH₂O
- **5x Loading buffer for Western Blot:** 220 mM Trizma[®] base, 22.5 mM EDTA, 9% (w/v) SDS, 1.8 M sucrose, 0.12 mg/ml bromphenolblue, dH₂O
- **TBS-T:** 150 mM NaCl, 0.1% (w/v) Tween 20, dH₂O, pH adjusted to 7.6
- **Resolving gel:** 3.75 ml buffer A, 7.5 ml 30% acrylamide, 300 μl 10% SDS, 9 ml 2 M sucrose, 9 ml dH₂O, 23 μl TEMED, 375 μl 25mg/ml APS
- **Stacking gel:** 1.875 ml buffer B, 1.875 ml 30% acrylamide, 150 μl 10% SDS, 3.75 ml 2 M sucrose, 7 ml dH₂O, 7.5 μl TEMED, 480 μl 25mg/ml APS
- **Buffer A for resolving gel:** 1M HCl, 3M Trizma[®] base, dH₂O, pH adjusted to 8.9
- **Buffer B for stacking gel:** 1.75 ml 85% ortho-phosphoric acid, 470 mM Trizma[®] base, dH₂O, pH adjusted to 6.7

3.1.4 Tested drugs

Table 4

Substances	Company and cat. #	Conc. used
Amoxicillin	Applichem Pancreac, A7680	300 μ M
Bezafibrate	Sigma, B7273	200 μ M
Boldine	Sigma, B3916 Fluka	10 μ M
Bupropion	Alfa Aesar, J61105	10 μ M
Chlorpromazine	Sigma, C8138	5 μ M
Cyclosporin A	Sigma, C1832	1 μ M
Clavulanic acid	Molekula, 8744048	100 μ M
Cynarin	Sigma, D8196	100 μ M
Fenofibrate	Sigma, F6020	200 μ M
Fluconazole	Sigma, PHR1160	10 μ M
Itraconazole	Sigma, I6657	10 μ M
Ketoconazole	Sigma, K1003	10 μ M
Luteolin	Sigma, L9283	10 μ M
Nefadozone	Alfa Aesar, J62793	10 μ M
Octreotide	Abbiotec, 350305	25 μ M
Olanzapine	Sigma, O1141	10 μ M
Posaconazole	Sigma, 32103	10 μ M
PSC833	Tocris, 4042	4 μ M
Verapamil	Sigma, V4629	10 μ M
Voriconazole	Sigma, PZ0005	10 μ M

3.1.5 Primary antibodies

Table 5

Antibodies	Supplier and cat. #	Host	Clone	Dilution*
α -ABCG5	Abcam, ab124965	rabbit	monoclonal	1:1000 WB
α -alpha 1 Na-K-ATPase	Abcam, ab7671	mouse	monoclonal	1:100 IF
K36, α -hABCG8	Own lab	rabbit	polyclonal	1:2000 WB
K37, α -hBSEP	Own lab	rabbit	polyclonal	1:500 WB 1:35 IF
K9, α -hNTCP	Own lab	rabbit	polyclonal	1:1000 WB 1:300 IF
P-glycoprotein (P3II-26), α -hMDR3	Enzo, ALX-801-028	mouse	monoclonal	1:600 WB 1:35 IF
Pan Actin (ACTN05 C4)	NeoMarkers, MA5-11869	mouse	monoclonal	1:1600 WB
P-glycoprotein (C219), α -MDR1/3	Enzo, ALX-801-002	mouse	monoclonal	1:100 WB
P-glycoprotein (C494), α -hMDR1	Enzo, ALX-801-003	mouse	monoclonal	1:100 WB

*Dilutions are shown for immunofluorescence (IF) or Western Blotting (WB).

3.1.6 Secondary antibodies

Table 6

Antibodies	Supplier and cat. #	Host	Clone	Dilution*
Alexa Fluor [®] 488 conjugate α -rabbit	Life technologies, A11008	goat	polyclonal	1:300 IF
Alexa Fluor [®] 568 conjugate α -mouse	Life technologies, A-11004	goat	polyclonal	1:300 IF
ECL α -mouse IgG horseradish peroxidase	GE Healthcare, NA931V	sheep	polyclonal	1:3000 WB
Goat α -rabbit IgG horseradish peroxidase	GE Healthcare, RPN4301	goat		1:30000 WB

*Dilutions are shown for immunofluorescence (IF) or Western Blotting (WB).

3.2 Methods

3.2.1 Maintenance of LLC-PK₁ cells

Wild type and stably transfected LLC-PK₁ cell lines were already available in the laboratory of Clinical Pharmacology and Toxicology at the start of the project. Transfection of the cell lines has been described in our recent publication (Mahdi et al., 2016). LLC-PK₁ cell lines (WT: wild type; BN: NCTP, BSEP; BNMG: NCTP, BSEP, MDR3 and ABCG5/G8) were cultured in high glucose (4.5g/L) Dulbecco's modified eagle's medium or DMEM (cat.# 21969035, Gibco, Paisley, UK), supplemented with 5% (v/v) fetal calf serum or FCS of US origin (cat.# 26140079, Gibco, Paisley, UK), 100 Units/ml penicillin, 100 µg/ml streptomycin (cat.# 15140122, Gibco, Grand Island, NY, USA) and 2 mM L-glutamine (cat.# 25030024, Gibco, Paisley, UK) at 37°C in a 5% CO₂ humidified atmosphere. The medium of the transfected control cell line, BN, was additionally supplemented with 700 µg/ml geneticin sulfate G418 (cat.# 11811031, Gibco, Grand Island, NY, USA) and 2 µg/ml puromycin (cat.# P7255, Sigma, MO, USA). The medium of the multi-transfected model cell line, BNMG, was additionally supplemented with 700 µg/ml geneticin sulfate G418, 400 µg/ml hygromycin B (cat.#14816600, Boehringer Ingelheim, Mannheim, Germany), 2 µg/ml puromycin and 20 µg/ml zeocin (cat.# 460509, Invitrogen, Carlsbad, CA, USA). Cells were passaged twice per week once they reached 80% to 100% confluency in 10 cm cell dishes. To split cells, the growth medium was removed and cells were washed once with 1x Dulbecco's Phosphate-Buffered Saline or PBS (cat.# 14200067, Gibco, Paisley, UK) at room temperature. 1 ml trypsin-EDTA (cat.# 25300062, Paisley, UK) was added and quickly removed. Cells were detached by adding 3 ml trypsin-EDTA and incubating for 5 min, at 37°C. To stop the enzymatic reaction, 7 ml complete/standard growth medium were added to the trypsin buffer. Cells were split at ratios ranging between 1:5 to 1:15 (WT: 1:10-1:15, BN and BNMG: 1:5-1:10).

3.2.2 Maintenance of Caco-2 cells

Caco-2 cells were cultured in high glucose DMEM (cat.# 11960044, Gibco, Paisley, UK), supplemented with 5% (v/v) FCS of US origin and 100 Units/ml penicillin and 100 µg/ml streptomycin. Cells were kept at 37°C in a 5% CO₂ humidified atmosphere and passaged twice per week once they reached 90% confluency.

3.2.3 Freezing and thawing of cells

Cells from an 80 to 100% confluent 10 cm dish were trypsinized and resuspended in complete/standard growth medium. To remove the medium, cells were centrifuged at 250 g for 5 min in an Eppendorf Centrifuge 5810R and the supernatant was aspirated using a sterile Pasteur pipette. The cell pellet was dissolved into 3 ml freezing medium consisting of 20% FCS, 10% DMSO and

70% standard/complete growth medium and aliquoted into cryotubes (1 ml each). The cryotubes were stored at -80°C into a freezing container to allow slow freezing. After 2-5 days the cryotubes were transferred to a liquid nitrogen tank for long term storage.

Cells were quickly thawed by holding a cryotube in a water-bath at 37°C and successively resuspended in 10 ml pre-warmed medium. The cell suspension was centrifuged at 250 g for 5 min. and the supernatant was discarded to remove DMSO. Finally the cell pellet was resuspended in 10 ml complete medium and plated on a 10 cm petri dish. 1 day later the growth medium was replaced to remove residual DMSO.

3.2.4 Cell counting

10 µl of Trypan Blue stain 0.4% (cat.# T10282, Invitrogen, Eugene, OR, USA) were added to 10 µl of cell suspension in an Eppendorf tube. After mixing, 10 µl of the sample mixture were pipetted into a cell counting chamber slide (cat.# C10228, Invitrogen, Eugene, OR, USA). Cells were counted using the CountessTM automated cell counter (Invitrogen, Eugene, OR, USA).

3.2.5 Culturing LLC-PK₁ cells in the Transwell[®] system

To investigate vectorial transport of lipids and bile salts, LLC-PK₁ cells were cultured on 12-wells Transwell[®] plates (cat.# 3402, Corning, NY, USA) having polycarbonate membrane inserts with a pore size of 3 µm. One day before cell seeding, Transwell[®] inserts were coated with 30 µl of a 1:4 diluted (in 50% ethanol) collagen R (cat.# 47254, SERVA, Heidelberg, Germany) solution and dried at room temperature. Cells were seeded on Transwell[®] membrane inserts at densities of 1.5×10^5 to 2×10^5 cells/insert and grown for 10-11 days. The medium in both compartments (0.5 ml apical and 1.5 ml basolateral) was carefully replaced every 4 days taking care to not to harm the monolayers of cells.

3.2.6 Lucifer yellow monolayer integrity test

One day prior performing transport experiments, the integrity of cell monolayers was tested using the fluorescent compound Lucifer yellow (cat.#80015, Biotium, Hayward, CA, USA) (Stewart, 1978). Apical and basolateral media were removed and replaced by a 100 µM Lucifer yellow solution (in DMEM) in the basolateral/donor compartment and DMEM without Lucifer yellow solution in the apical/acceptor compartment. Transwell[®] plates were slowly shaken and incubated for 1 h at 37°C in the cell incubator. 200 µl media aliquots were collected from the apical compartments and the amount of Lucifer yellow was determined by measuring the fluorescence in a Twinkle LB 970 Microplate Fluorometer at λ_{ex} 430 nm, λ_{em} 535 nm (Stewart, 1981). Permeability of Lucifer yellow was calculated as percent of Lucifer yellow in the receiver apical compartment as compared to the control (well containing only filters with no cell monolayer). Only monolayers with a permeability of less

than 5% were selected for experiments. After the integrity test, the medium was removed and replaced with standard culture medium.

3.2.7 ^3H -taurocholate transport assay

Transport assays using the radioactively labelled bile salt taurocholate were performed to demonstrate functionality of the bile salts transporters NTCP and BSEP. For this, DMEM solution containing 10 μM taurocholate (final concentration) was prepared by dissolving ^3H -taurocholate (at 0.2 $\mu\text{Ci/ml}$) and unlabeled taurocholate in DMEM and pre-warmed in a water bath at 37°C. After removing the standard growth medium in both compartments, the taurocholate containing DMEM was added either to the basolateral or to the apical compartment and pre-warmed serum free DMEM was added to the corresponding opposite/acceptor compartment. After 10, 20 and 30 min 50 μl aliquots were collected from the acceptor compartments and transferred to scintillation vials. Moreover, to assess intracellular accumulation of taurocholate, 50 μl aliquots of the solubilized monolayers (as described below) were also transferred to scintillation vials. To each vial 4 ml of Filter-count liquid scintillation cocktail was added. Radioactivity in the aliquots of medium or solubilized monolayers was determined by liquid scintillation counting using a Canberra Packard Tri-Carb 2250CA liquid scintillation analyzer.

3.2.8 Testing the effect of drugs on the bile salts transporters, BSEP and NTCP

Overnight treatment of cell monolayers with drugs (10 μM azoles or 4 μM PSC833) was followed by a taurocholate transport assay from the basolateral to the apical compartment (as previously described). Cell monolayers were treated for 24 h with the drugs added to both compartments before performing the taurocholate transport assay. Aliquots from both the apical medium and cell lysates were analyzed for amount of ^3H -taurocholate.

3.2.9 Cholesterol efflux assay

DMEM containing 1 μM ^{14}C -cholesterol (0.5 $\mu\text{Ci/ml}$) and 1 mM taurocholate and DMEM containing 50 mg/ml fatty acid free albumin were prepared and pre-warmed in a water bath at 37°C. Apical and basolateral culture media were removed and replaced by 1.5 ml DMEM containing the radiolabeled cholesterol in the basolateral/donor compartment and 0.5 ml DMEM containing 50 mg/ml fatty acid-free bovine serum albumin in the apical/acceptor compartment to stimulate lipid efflux. After 24 h 50 μl aliquots were collected from the apical compartments and transferred to scintillation vials. To each vial 3 ml of Filter-count liquid scintillation cocktail was added. Radioactivity in the aliquots of medium or solubilized monolayers was determined by liquid scintillation counting using a Canberra Packard Tri-Carb 2250CA liquid scintillation analyzer.

3.2.10 Phosphatidylcholine efflux assay

In order to check for functionality of MDR3, phosphatidylcholine efflux assays were performed. For these, apical and basolateral culture media were removed and replaced by 1.5 ml DMEM containing 1 mM taurocholate in the basolateral/donor compartment, to mimic the physiological situation, and 0.5 ml DMEM containing 50 mg/ml fatty acid-free bovine serum albumin (cat.# A6003, Sigma, MO, USA) in the apical/acceptor compartment to ensure solubility of the translocated lipids. After 24 h the apical medium was collected and lipids were extracted (as described below). Extracted lipids were loaded on HPTLC Silica gel plates and separated by Thin-Layer-Chromatography (TLC).

3.2.11 Lipid extraction

Lipids were extracted according to the Bligh and Dyer method (Bligh and Dyer, 1959) using a chloroform/methanol/water ratio of 1:2:0.8. 400 μ l of apical medium samples were pipetted in extraction glass tubes and 1.5 ml methanol/chloroform 2:1 ml was added to each sample. After shaking 20 min at 350 rpm and room temperature, samples were transferred to clean glass tubes to remove precipitated proteins. Subsequently, 0.5 ml 0.1 M KCl was added to each tube and tubes were shortly vortexed. Next, tubes were centrifuged 5 min at 3000 rpm (1810 g) in an Eppendorf Centrifuge 5810R to separate the phases. After centrifugation two phases, the lower corresponding to the chloroform-lipid phase and the upper corresponding to the methanol-water phase were clearly visible.

For analysis of secreted phosphatidylcholine by TLC the upper phase was discarded, the lower-lipid phase was evaporated using Nitrogen gassing in a warming block at 37°C and lipids were resuspended in 125 μ l chloroform. Samples were aliquoted in glass vials and stored at -20°C. For analysis of secreted C₆-NBD-PC 300 μ l of the lower-lipid phase were directly used for assessing C₆-NBD-PC content by measuring fluorescence.

3.2.12 Lipid analysis by Thin-Layer-Chromatography

For analysis of the phospholipid or cholesterol content in the apical medium, extracted lipids were loaded on HPTLC Silica gel 60 plates with concentrating zone (cat.# 113749, Merck KGaA, Darmstadt, Germany) using an automated Camag TLC sampler ATS4 and separated by one-dimensional Thin-Layer-Chromatography (TLC) as previously described (Gerloff et al., 1998). 30 ml eluting solvent composed either of methylacetate, 1-propanol, chloroform, methanol and 0.5% potassium chloride in a 25:25:25:10:9 (v/v) ratio for phospholipids, or of n-hexane, n-heptan, diethylether and acetic acid in a 63:18.5:18.5:1 (v/v) ratio for the analysis of cholesterol, was poured in a twin trough chamber (10 ml in the front and 20 ml in the back). A saturation pad was placed in the back and the chamber was closed and saturated for 15 min. Next, the TLC plate was carefully placed in the chamber with the solvent level below the level of the loaded samples. The plate was

developed for about 25 min, until the solvent migrated to about 0.5 cm below the top of the plate. After air drying at room temperature, plates for the phospholipids analysis were developed again following the same procedure. Finally the plate was dried in a drying-oven at 100°C for 10 min. To visualize the lipids, the plate was stained by dipping it for 15 s in a 3% (w/v) copper acetate 8% (v/v) ortho-phosphoric acid solution and heating at 110°C for 15 to 25 min in a drying-oven. Bands were quantified as previously described (Gerloff et al., 1998) by densitometry with a Camag TLC Scanner 3 using a standard curve for each phospholipid which was loaded on the same plate.

3.2.13 C₆-NBD-PC efflux assay

Alternatively to the phosphatidylcholine efflux assay, MDR3-mediated phospholipid secretion was assayed using the fluorescent phosphatidylcholine derivative C₆-NBD-PC (1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4yl)amino]hexanoyl}-sn-glycero-3-phosphocholine, Fig. 5). Experiments were started by replacing the apical medium with DMEM supplemented with 50 mg/ml fatty acid free albumin and the basolateral medium with DMEM containing 12.5 μ M C₆-NBD-PC and 1 mM taurocholate. After 24 h the apical medium was collected and lipids were extracted as described above. Fluorescence of the lipid containing chloroform phase was measured at λ_{ex} 485 nm and λ_{em} 535 nm, with counter position at the top, in a Twinkle LB 970 Microplate Fluorometer (Berthold Technologies, Bad Wildbad, Germany). For quantification, standards of C₆-NBD-PC dissolved in albumin-supplemented DMEM were extracted in parallel to the samples.

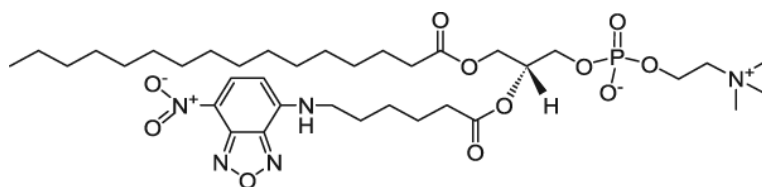


Fig. 5 Chemical structure of C₆-NBD-PC. Obtained from Avanti Polar Lipids Inc., US (avantilipids.com, 2016)

3.2.14 Testing the effect of drugs on MDR3 function using the C₆-NBD-PC efflux assay

C₆-NBD-PC efflux assay was carried out as described above in presence of the tested drugs which were added to both compartments during the assay (DMSO final concentration did not exceed 0.5% v/v). Drug concentrations were chosen considering the maximal total plasmatic concentrations reported in the literature and *in vitro* cell cytotoxicity. After 24 h, the apical medium was collected, lipids were extracted and fluorescence was measured.

3.2.15 Validating the effect of drugs on MDR3 function using the ¹⁴C labelled choline

Cell monolayers were pre-incubated 4 h in minimal essential medium (MEM cat. 4655) containing 0.3 μ M ¹⁴C-choline chloride (0.6 μ Ci/ml). After removing the medium, cell monolayers were washed

once with 1xPBS and incubated overnight in DMEM containing 1 mM taurocholate in the basolateral compartment and DMEM containing 50 mg/ml fatty acid free albumin in the apical compartment. The tested drug was added to both compartments (2 or 10 μ M itraconazole). After overnight incubation (10 h) the apical medium was extracted and the lipid phase was loaded on a HPTLC plate and developed as previously described for the phospholipids. To visualize the radioactively labelled phospholipids secreted in the apical compartment (14 C-PC), an X-ray film was exposed to the HPTLC plate in a tight, dark cassette for 8 days. The film was developed using a medical film processor (Fujifilm). Finally to visualize all the phospholipids, including the standards, on plate, the HPTLC plate was stained as described before by dipping in a 3% (w/v) copper acetate 8% (v/v) orthophosphoric acid solution and heating for 15 to 25 min at 110°C in a drying-oven.

3.2.16 Cytotoxicity assay

Potential cytotoxicity of the drugs used was checked by measuring LDH release in the apical and basolateral medium. This was performed 24 h after drug supplementation using a CytoTox96 Non-Radioactive Assay Kit (cat.# G1780, Promega, Madison, WI, USA). 50 μ l of basolateral, apical medium and the cell lysate were pipetted into a well of a 96-well plate. As the LDH amount is proportional to the amount of cells, the LDH released in the medium was normalized to the LDH in the corresponding lysate. To each well 50 μ l of reconstituted substrate mix were added. The plate was incubated for 30 min at room temperature in the dark. Finally, 50 μ l stop solution were pipetted to each well and absorption at 490 nm was measured using a Bio-Rad Ultramark Microplate Reader.

3.2.17 Solubilization of cell monolayers

Transport assays were normalized to the amount of protein per monolayer. To solubilize cell monolayers for protein determination, Transwell® filters were rinsed with PBS, cut out of the Transwell® insert using a scalpel and transferred to labelled Eppendorf tubes containing 200 μ l 1% (w/v) Triton X-100 on ice. To detach the cells from the filters, the tubes were centrifuged at 4°C at full speed for 40 min. in an Eppendorf Centrifuge 5417R (Hamburg, Germany).

3.2.18 Protein determination

The protein amount was determined by the bicinchononic acid method (Smith et al., 1985) using the BCA protein assay kit (Interchim, Montluçon, France). 25 μ l of cell lysate was transferred to a 96-well microplate and diluted with 25 μ l of distilled water. In parallel, 25 μ l of bovine serum albumin standards (0, 0.025, 0.125, 0.25, 0.5, 0.75, 1, 1.5 mg/ml in water) were transferred on the same plate and diluted with 25 μ l of sample's solvent (1% Triton X-100). 200 μ l of a BCA kit reagent solution was prepared and added to each well. After 30 min. incubation of the plate at 37°C, absorption at 550 nm was measured in a Bio-Rad Ultramark Microplate Reader (Hercules, CA, USA).

3.2.19 Isolation of cell membranes

To isolate the total membrane fraction of WT, BNMG and Caco-2 cells, 10 cm cell dishes at 100% confluency were rinsed three times with 10 ml 0.9 % (w/v) NaCl and once with 10 ml 250 mM sucrose. After completely aspirating the 250 mM sucrose solution, 3 ml of ice cold 5 mM sucrose solution containing 5 mM PMSF and 1 g/ml antipain/leupeptin (protease inhibitors) was/were added to each dish and cells were scraped with a rubber policeman. All the following steps were conducted at 4°C to avoid protein degradation. The cell suspension was transferred into a tight fitting glass-teflon potter on ice. Residual cells were collected by rinsing the dish again with 1 ml, 250 mM sucrose and transferring the rinse to the suspension in the glass-teflon potter. The cell suspension in the glass-teflon potter was homogenized with 20 strokes at full speed with a machine driven pestle. The cell homogenate was transferred in the appropriate centrifuge tubes and centrifuged for 10 min at 2950 rpm (900 g) in a Sorvall RC5C centrifuge with a SS34 rotor. The resulting supernatant was transferred to clean tubes and centrifuged again for 20 min at 9600 rpm (8500 g). Next, the resulting supernatant was transferred to clean tubes and centrifuged for 1 h at 4°C at 38'200 rpm (100'000 g) in a Centrikon T-1170 ultracentrifuge with a TFT 65.13 rotor. Finally, the supernatant was discarded and the pellet was resuspended in 100 to 200 µl 250 mM sucrose using a 1 ml syringe with a thin needle. Protein concentration of the isolated membranes was determined with the BCA assay. Isolated membranes were stored at -80°C.

3.2.20 Western Blotting

To analyze protein samples by Western Blotting, proteins were separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Isolated membranes or whole cell lysates (of Transwell® monolayers or cells grown on 10 cm dishes) were denatured by adding 5X SDS loading buffer containing freshly added 15mg/ml DTT and incubating the samples for 20 min at 40°C. The samples were then loaded on a 7.5% SDS-PAGE maxi-gel. In order to compare protein expression levels, the same amount of protein was loaded for all samples on the same gel (60 to 100 µg/lane). The proteins were separated overnight in BioRad PROTEAN II xi Cell applying a potential of 6 mA per gel. Next, the separated proteins were transferred to a nitrocellulose membrane. For transferring the proteins, a cassette was assembled with sponges outside, followed by three Whatman papers and a nitrocellulose membrane and the SDS-PAGE gel in the middle (sandwich). Sponges, papers, and membrane were all pre-soaked in transfer buffer. The transfer was performed in a BioRad Trans-Blot® Cell filled with transfer buffer at an applied current of 300 mA for 4 h. Membranes were blocked 1 h in 5% (w/v) milk-TBS-T and then probed for human MDR3, BSEP, NTCP, ABCG5/G8, porcine MDR1 and actin by incubation for 2 h at room temperature with the appropriate first antibody diluted in 5% (w/v) milk-TBS-T. After washing three times in TBS-T for 10 min, blots were incubated for 1 h at room temperature with the appropriate secondary antibody, either an anti-mouse

horseradish peroxidase (HRP) or anti-rabbit HRP antibody. Proteins bands were visualized using the UptiLight chemiluminescence reagent. In a dark room, an X-ray film was exposed to the luminescent bands on the membrane and developed using a medical film processor (Fujifilm).

3.2.21 Stripping of Western blots

For stripping off antibodies, the nitrocellulose membrane was washed in TBS-T for 15 min and then incubated for 15 min in RestoreTM PLUS Western Blot Stripping Buffer (cat. # 46430, Thermo Scientific, Rockford, IL, USA). Finally the membrane was washed again three times for 10 min in TBS-T.

3.2.22 Quantitative Real-Time qRT-PCR analysis

BNMG cells were grown to confluency in 6-well plates and treated for 24 h with 10 μ M itraconazole or 0.1% (v/v) DMSO. To extract total RNA, cells were lysed in 0.5 ml/well TRIzol[®]. Cell lysates were transferred to Eppendorf tubes and 100 μ l chloroform were added. Tubes were shaken, incubated for 2-3 min at room temperature and then centrifuged in Eppendorf Centrifuge 5417R at 12'000 g and 4°C for 15 min to induce separation of the phases. The upper phase containing only RNA was transferred into clean Eppendorf tubes. 250 μ l isopropanol was added to each sample. Tubes were incubated for 10 min at room temperature and then centrifuged again at 12'000 g for 10 min at 4°C. The supernatant was removed by decantation and the RNA pellet was washed by adding 80 μ l of 75% ethanol followed by centrifugation at 7'500 g for 5 min at 4°C. The supernatant was carefully aspirated and the RNA pellet was air dried for about 5-10 min. Finally, the pellet was resuspended in 20 μ l RNase-free water and stored at -80°C. The RNA concentration was determined using the spectrophotometer NanoDrop ND-1000. To reverse transcribe RNA to cDNA, 1 μ g RNA was pipetted in Eppendorf tubes and RNase-free water was added to reach a total volume of 10 μ l. Then, 10 μ l of TaqMan[®] Fast Universal PCR Master Mix were added to each tube, samples were vortexed and incubated for 1 h at 37°C. To stop reverse transcription, the samples were incubated for 5 minutes at 85°C. The cDNA was diluted by adding 60 μ l of RNase-free water and subsequently stored at -20°C. To quantify mRNA levels of MDR3 and GAPDH by qPCR, cDNA (2 μ l) was amplified using TaqMan master mix (8 μ l) and human MDR3 primer (Hs00240956_m1) or GAPDH primer (Ss03374854_g1) which were pipetted in triplicates in a 384-well plate and heated once to 95°C followed by 40 cycles heating for 1 s at 95°C and for 20 s at 60°C. qPCR was performed using an AB applied biosystems ViiA7 (Life technologies, USA). mRNA levels were expressed relative to the housekeeping gene GAPDH.

3.2.23 Immunofluorescence

To demonstrate polarized membrane localization of the transporters, immunostaining of cell monolayers grown on Transwell® filters was performed with fluorescently labelled antibodies. For this, the apical and basolateral medium were removed, Transwell® filters were rinsed with PBS and cut out of the Transwell® inserts using a scalpel and transferred to a 12-well plate. The monolayers were fixed by incubation for 20 min in 4% paraformaldehyde at room temperature. After three times washing with PBS, monolayers were incubated for 5 min in 0.25% NH₄Cl in PBS to quench the free aldehyde groups of the paraformaldehyde fixative. In order to permeabilize the cells to allow the antibodies to cross cellular membranes, all next steps were performed in presence of 0.1% saponin. Cell monolayers were incubated for 10 min in PBS containing 0.1% (w/v) saponin and then for 30 min in blocking solution consisting of 0.1% (w/v) saponin and 2% (v/v) goat serum in PBS. Subsequently, cells were washed twice 10 min with PBS containing 0.1% (w/v) saponin and 1% (w/v) bovine serum albumin. Finally, cells were incubated with the primary antibody recognizing the antigen of interest for 2 h, washed 3 times and incubated with the fluorescently labeled secondary antibody recognizing the primary antibody for 1 h. Antibodies were diluted in PBS containing 0.1% (w/v) saponin and 1% (w/v) bovine serum albumin. After rinsing the cells three times with PBS, filters were mounted with one drop Vectashield mounting medium (with DAPI) between a microscope slide and a coverslip, with the apical side facing the coverslip and the basolateral side/filter facing the microscope slide. Coverslips were fixed with nail polish to the microscope slide and stained monolayers were stored in the dark at 4°C.

3.2.24 Confocal laser scanning microscopy

For confocal microscopy the Leica SP8 confocal microscope with the objective HCX PL APO CS2 (magnification of 63x, NA 1.4, oil, WD 0.14) was used. Pictures were exported and processed in ImageJ.

3.2.25 Statistical Analysis

Data are expressed as mean \pm SD. Paired t-test was used to evaluate the difference between experimental and control absolute values and One sample t-test was used to evaluate the difference between experimental and control values of normalized data. The statistical analysis was carried out with GraphPad Prism (GraphPad Software Inc., San Diego, USA). Differences with $p < 0.05$ were considered significant.

4 Results

4.1 Characterization of a model cell line for biliary lipid secretion

In the first part of this project, the model LLC-PK₁ cell line stably transfected with NTCP, BSEP, MDR3 and ABCG5/G8, all under a constitutive CMV promoter was characterized. This cell line was named BNMG and expression and function of the respective transporters were tested using various assays.

4.1.1 Western blot analysis of cell lines

Expression of the transfected transporters in BNMG cells was assessed by Western Blotting Fig. 6). NTCP, BSEP, MDR3 and ABCG8, as well as ABCG5 were detected in BNMG cells, but not in WT cells. In the BN control cell line (stably transfected with BSEP and NTCP) only BSEP and NTCP were detected.

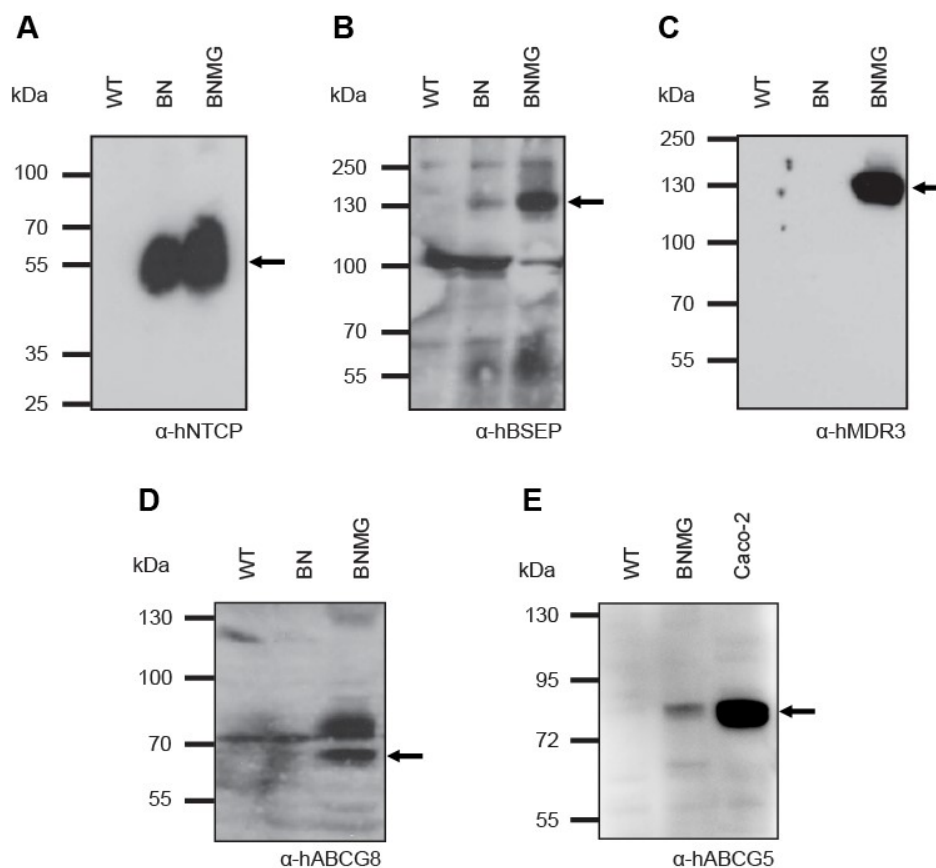


Fig. 6 Western blot analysis of LLC-PK₁ cell lines. Presence of (A) NTCP, (B) BSEP, (C) MDR3, (D) ABCG8; (E) ABCG5 in BNMG cells was assessed by Western Blotting. In (A) to (D) lanes were loaded with 75 µg protein of WT: wild type cells, BN: control cell line transfected with NTCP and BSEP and BNMG: model cell line transfected with NTCP, BSEP, MDR3 and ABCG5/G8 total cell lysates. In (E) lanes were loaded with 70 µg of a total membrane fraction isolated from WT, BNMG or Caco-2 cells.

4.1.2 Immunolocalization of BSEP, MDR3 and NTCP in polarized LLC-PK₁ cells

To assess the polarized expression of NTCP, BSEP and MDR3 in BNMG cells, immunohistochemical analysis of BNMG cells was carried out. Cells were grown 10 days on Transwell® filters, fixed and double immunostained, against MDR3 and NTCP or against BSEP and the endogenous Na⁺/K⁺-ATPase. Fig. 7 shows top view images of BNMG monolayers taken at different z-stacks using a confocal laser scanning microscope. MDR3 and BSEP were detected at about 7-8 µm from the Transwell® filters. In contrast, NTCP and the endogenous Na-K-ATPase, which are known to be expressed on the basolateral membrane of LLC-PK₁ cells (Liu et al., 2004), were detected closer to the Transwell® filters, at 3-4 µm.

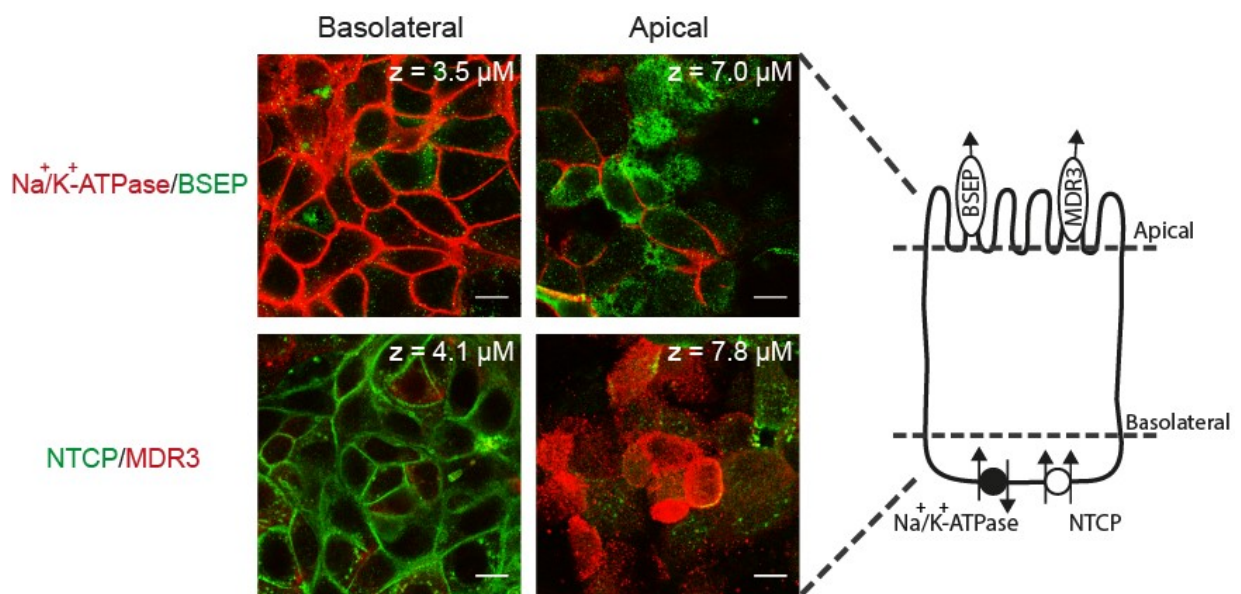


Fig. 7 Confocal immunofluorescence images of BNMG cells at different Z-planes. BNMG cells were grown to monolayers on Transwell® filters. Representative XY-images at lower (basolateral) and upper (apical) Z-planes are shown. The Na⁺/K⁺-ATPase and MDR3 are shown in red. BSEP and NTCP are shown in green. Scale bars: 10 µm.

In addition, results of the immunohistochemical analysis were further complemented with orthogonal projections of the total z-stacks in Fig. 8.

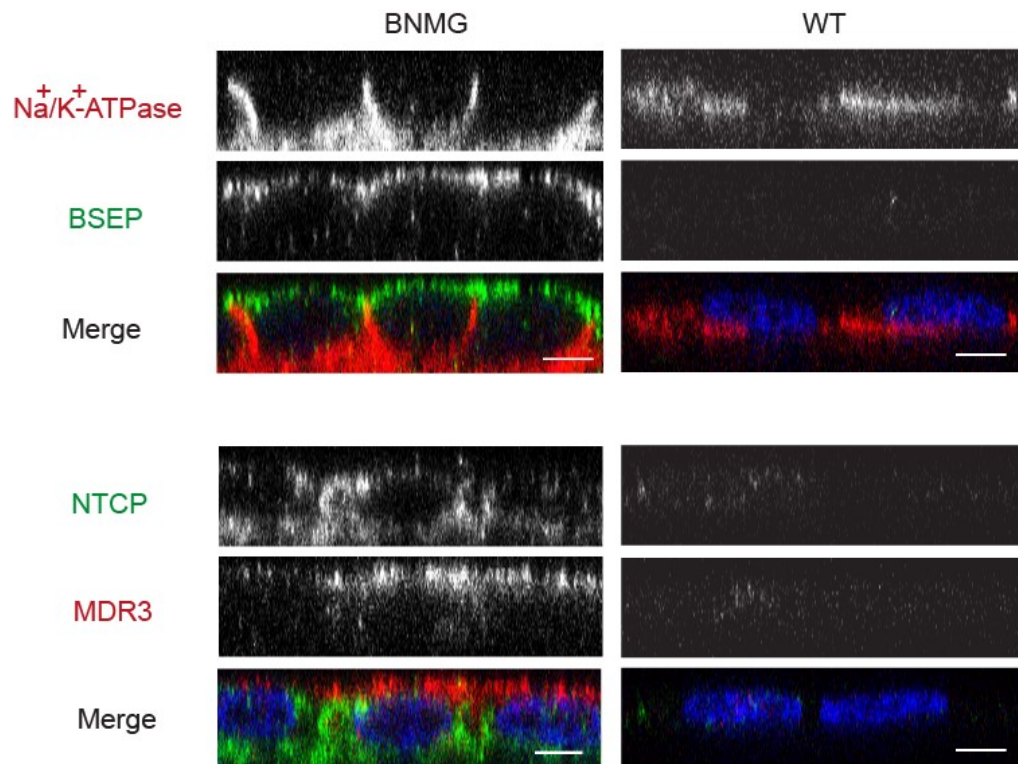


Fig. 8 Orthogonal projection of z-stacks of WT and BNMG cells. BNMG cells grown to monolayers on Transwell® filters and immunostained against Na⁺/K⁺-ATPase and BSEP or NTCP and MDR3 are shown. Nuclei were stained with DAPI and are shown in blue. Scale bars: 5 μ m.

Hence, Fig. 7 and Fig. 8 confirm polarized expression of BSEP, MDR3 and NTCP, as is the case in human hepatocytes (Pauli-Magnus and Meier, 2006). BSEP and MDR3 are expressed on the apical domain, whereas NTCP is expressed on the basolateral domain. WT cells do not show staining for BSEP, MDR3 and NTCP suggesting that the staining was specific.

4.1.3 Assessment of integrity of the monolayers

To assess protein-mediated transport across cell monolayers, an important prerequisite is that the monolayers are tight; otherwise leakage would confound the measurements. Integrity of cell monolayers on Transwell® inserts can be assessed by measuring the leakage of a non-transportable fluorescent compound such as Lucifer yellow (Hidalgo et al., 1989). In preliminary experiments, tightness of monolayers was assessed over 6 days. Data in Fig. 9 show that, as expected, after cell seeding on Transwell® inserts, leakage of Lucifer yellow constantly decreased over 6 days, parallel to cell growth. Moreover, leakage was similar from the basolateral to the apical compartment and from the apical to the basolateral compartment, suggesting that the polarized transporters, expressed in BNMG cells, did not transport Lucifer yellow. To perform transport experiments, the upper threshold for Lucifer yellow permeability was set at 5%.

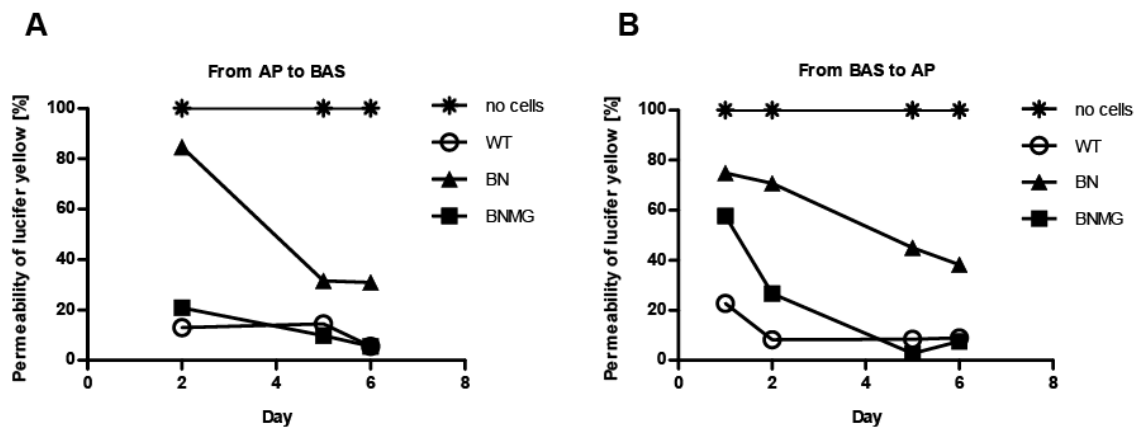


Fig. 9 Assessment of tightness of cell monolayers using Lucifer yellow. Lucifer yellow was added to a donor compartment and leakage was determined by measuring fluorescence of the medium in the opposite compartment after 1 h incubation. Permeability of Lucifer yellow was calculated as the ratio of the amount of Lucifer yellow in the receiver compartment compared to the maximal possible amount of Lucifer yellow (measured in absence of cell monolayers). (A) Lucifer yellow was added to the apical compartment and measured in the basolateral compartment. (B) Lucifer yellow was added to the basolateral compartment and measured in the apical compartment. Results of one experiment are shown.

4.1.4 Assessment of functionality of the bile salts transporters, NTCP and BSEP

Functional polarity of the bile salt transporters was assessed by comparing transcellular transport of the radioactively labelled bile salt taurocholate from the basolateral to the apical compartment with the transport from the apical to basolateral compartment. Fig. 10A and B show that the transport rate of taurocholate from the basolateral to the apical compartment is 56-fold higher than the transport from the apical to basolateral compartment. The corresponding ratio for WT cells is one.

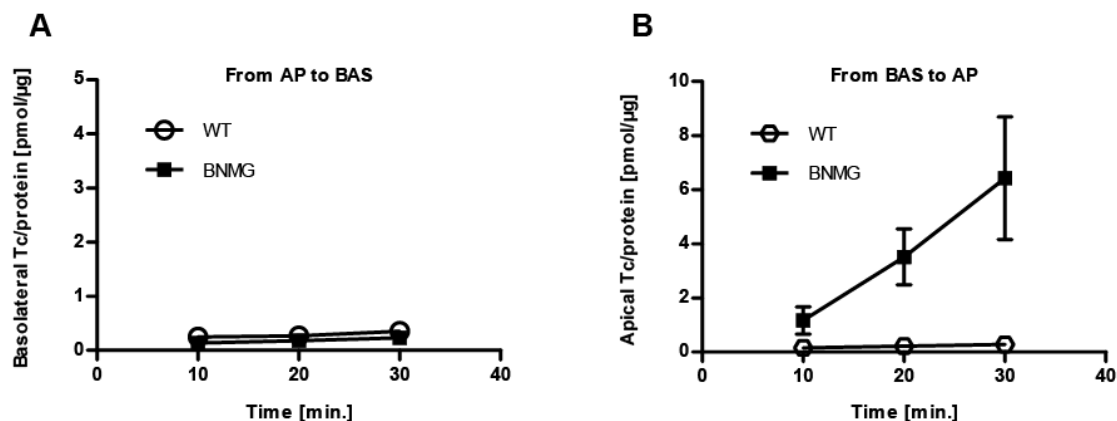


Fig. 10 Vectorial transport of taurocholate in BNMG cells. Transcellular transport of taurocholate in WT and BNMG cells was assessed in the Transwell® system. (A) Transport of 10 μ M taurocholate from the apical to the basolateral or (B) from the basolateral to the apical compartment was assayed in WT and BNMG cells. Results are shown as means of 3 independent experiments performed in triplicates \pm SD.

Moreover, Fig. 11A illustrates that after 2 h the net intracellular amount of taurocholate was 3-fold higher in BNMG cells as compared to WT cells and occurred exclusively if taurocholate was added to the basolateral side. In addition Fig. 11B shows that within 30 min. after adding 10 μ M taurocholate in the basolateral compartment, there was no further increase of the intracellular taurocholate suggesting that an equilibrium was reached. Taken together, the results of Fig. 10 and Fig. 11 indicate that NTCP and BSEP are functionally active in BNMG cells and mediate vectorial transport of bile salts through the basolateral and apical membrane.

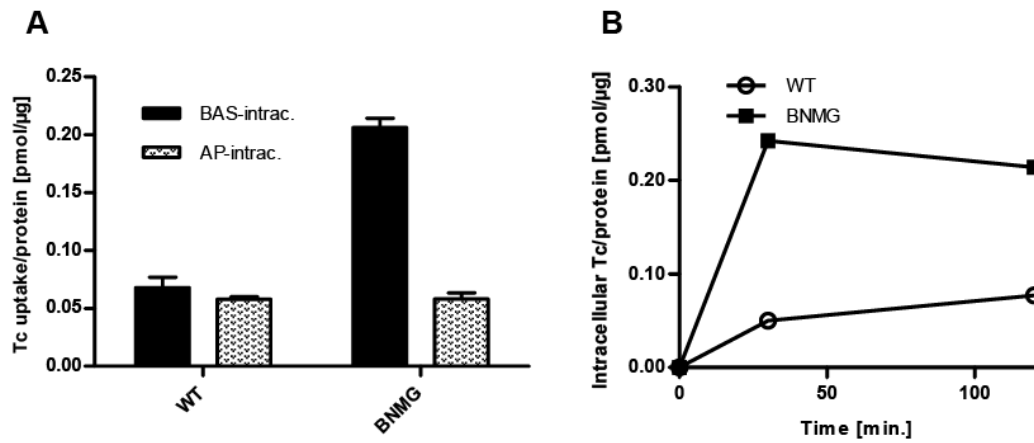


Fig. 11 Basolateral uptake of taurocholate in BNMG cells. (A) Intracellular accumulation of taurocholate was assessed 2 h after addition of 10 μ M taurocholate either to basolateral or to the apical compartment. Results are shown as means of 2 independent experiments performed in triplicates \pm SD. (B) The intracellular accumulation of taurocholate was measured at 30 min and at 2 h after adding 10 μ M taurocholate to basolateral compartment of BNMG cells. Results of one experiment performed in duplicates are shown.

4.1.5 Assessment of functionality of the cholesterol transporter ABCG5/G8

Several attempts were made to assess specific cholesterol transport in BNMG cells. Some representative examples of the tested conditions are shown in Fig. 12. In brief, supplementation of 1 mM taurocholate either in the basolateral or in the apical compartment had a minor effect on cholesterol secretion. In contrast, supplementation of 1 mM taurocholate to the basolateral and albumin or apolipoprotein A1 as acceptors to the apical compartments clearly increased by approximately 2-fold the amount of radioactively labelled cholesterol secreted in the apical compartment. However no significantly higher cholesterol secretion was observed in BNMG cells, suggesting the absence of ABCG5/G8-mediated cholesterol secretion. However no ABCG5/G8-mediated transport of cholesterol was observed in BNMG or at least not in a reproducible manner using these conditions.

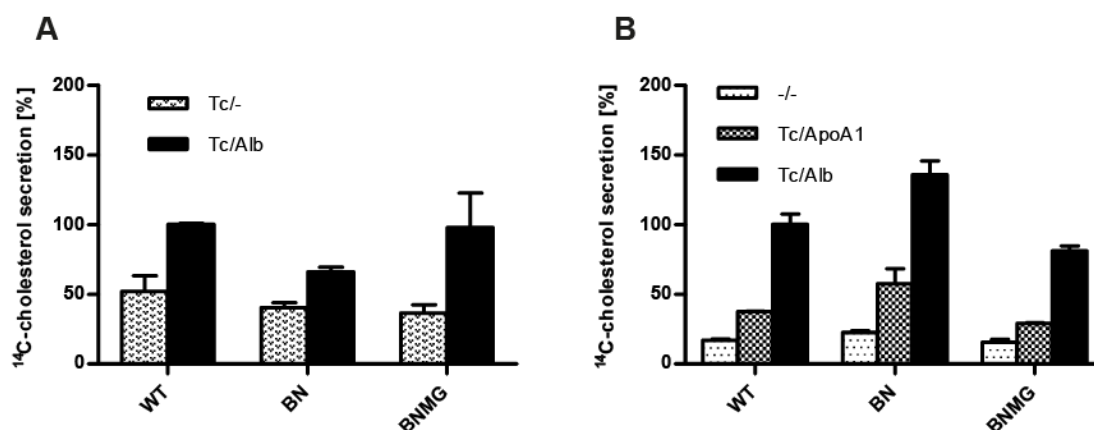


Fig. 12 ^{14}C -cholesterol secretion by BNMG cells. ^{14}C -cholesterol was added to the basolateral compartment and after 24 h apical ^{14}C -cholesterol secretion of WT, BN and BNMG monolayers was assessed. Data were normalized to the total amount of proteins per monolayer and presented in percent of WT (Tc/Alb). (A) ^{14}C -cholesterol secretion was measured either in the presence of only 1 mM taurocholate (Tc) basolateral or 1 mM taurocholate basolateral and 50 mg/ml albumin apical (Tc/Alb). Experiments are shown as means of 2 independent experiments performed in triplicates \pm SD. (B) Cholesterol secretion was measured in absence of any acceptor or taurocholate (-/-) or in the presence of 1 mM taurocholate in the basolateral compartment and either 10 $\mu\text{g/ml}$ ApoA1 or 50 mg/ml albumin to the apical compartment. A representative experiment is shown as means of 3 technical replicates \pm SD.

As LLC-PK₁ cells endogenously synthesize cholesterol, we hypothesized that endogenous cholesterol may have competed with the radiolabeled cholesterol and this may have decreased the secretion of the labelled substrate. Therefore, we also analyzed chemically the endogenous cholesterol secreted into the apical compartment with TLC (Fig. 13A). Similarly, as with the radiolabeled assay, supplementation of taurocholate to the basolateral, and albumin to the apical compartment, increased by approximately 2-fold endogenous cholesterol secretion. However, as shown in Fig. 13B

densitometric quantification of the HPTLC plates, indicated that cholesterol secretion in BNMG cells was not significantly higher than secretion by WT cells.

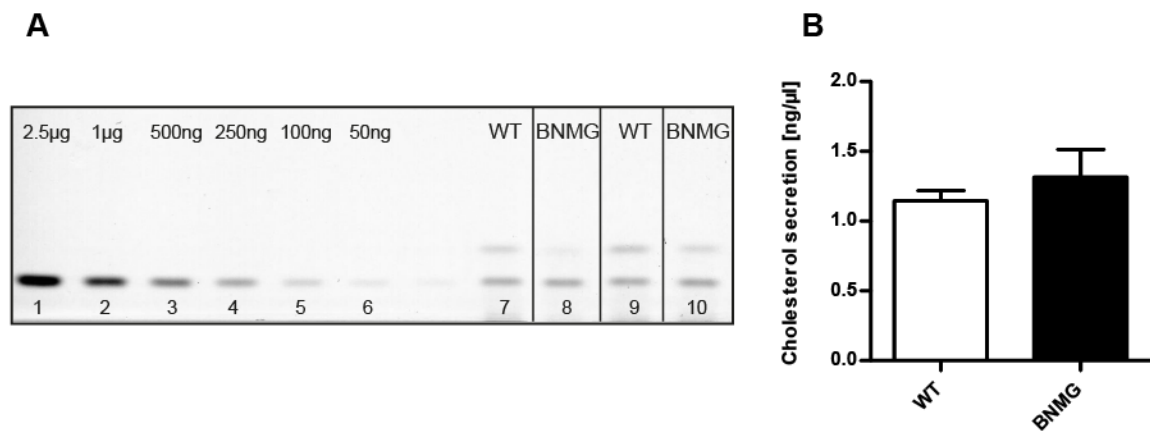


Fig. 13 Secretion of endogenous cholesterol by BNMG cells. Cell monolayers were treated with 1 mM taurocholate in the basolateral and 50 mg/ml fatty acid free albumin in the apical compartment, respectively. After incubation the apical medium was collected and processed to quantify the secreted cholesterol. (A) A representative HPTLC plate used for quantification of apical secreted cholesterol is shown. Lanes 1-6: Cholesterol standards; Lanes 7-8: cholesterol secreted after 14 h; Lanes 9-10 cholesterol secreted after 24 h. (B) Densitometric quantification of the secreted cholesterol after 24 h. Data are given as means of 2 independent experiments \pm SD, *P < 0.05.

4.1.6 Assessment of functionality of the phosphatidylcholine transporter MDR3

Smit and coworkers have shown that mice with a homozygous knockout of *mdr2* (MDR3 homologous gene in mice) completely lack phospholipid in their bile and develop liver disease (Smit et al., 1993). Subsequent experiments showed an increased appearance of C₆-NBD-PC in the exoplasmic leaflet of membranes of yeast expressing *mdr2* (Ruetz and Gros, 1994) as well as an increased secretion of ¹⁴C-PC in fibroblasts of transgenic mice expressing human MDR3, in the presence of a phospholipid acceptor (PC- Transfer Protein or PC-TP) in the medium (Smith et al., 1994). These data lead to the conclusion that MDR3 is a specific phosphatidylcholine (PC) translocase. Therefore, to assess functionality of MDR3 in BNMG cells we chemically analyzed with TLC, the phospholipids released into the apical compartment. To ensure the solubility and thus stimulate the release of the phospholipids in the medium cells were treated with 1 mM taurocholate in the basolateral and fatty acid free albumin in the apical compartment, respectively.

Fig. 14A shows that BNMG cells specifically secrete PC and that no other phospholipids could be detected. In addition, this figure indicates that the lipid acceptor albumin contains endogenous phosphatidylcholine, requiring correction of the secreted amount of PC. In Fig. 14B results of the densitometric quantification of HPTLC plates are shown. These data demonstrate that as compared to WT cells BNMG cells secrete a higher amount of PC into the apical compartment suggesting that MDR3 may be functionally active.

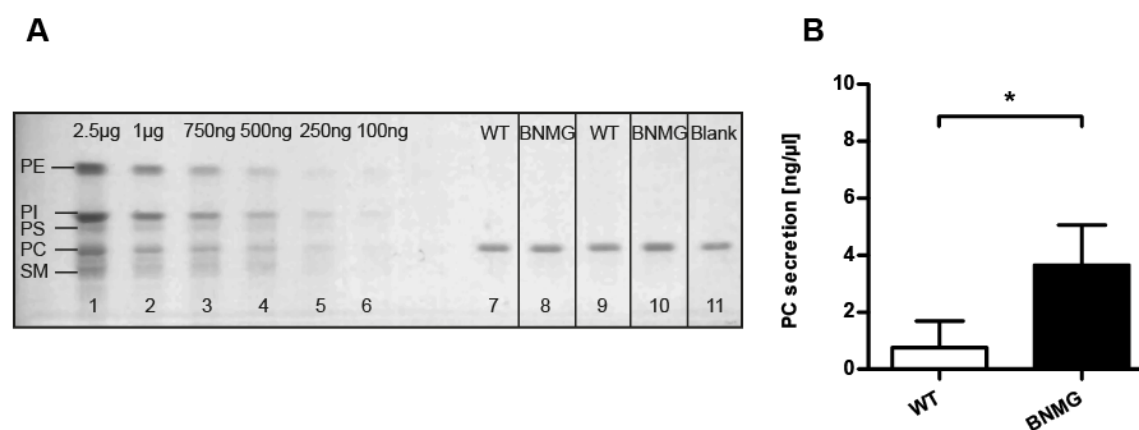


Fig. 14 Secretion of endogenous phosphatidylcholine by BNMG cells. Cell monolayers were treated with 1 mM taurocholate in the basolateral and 50 mg/ml fatty acid free albumin in the apical compartment, respectively. After incubation the apical medium was collected and processed to quantify the secreted phosphatidylcholine (PC). (A) A representative HPTLC plate used for quantification of apical secreted phospholipids is shown. Lanes 1-6: Phospholipid standards; Lanes 7-8: Phospholipids secreted after 14 h; Lanes 9-10 phospholipids secreted after 24 h; Lane 11: medium containing albumin. (B) Densitometric quantification of the secreted PC after 24 h. Data are corrected for albumin-bound PC (blank) and given as means of 4 independent experiments \pm SD, *P < 0.05.

We next tested the apical secretion of the fluorescent PC derivative C₆-NBD-PC, a known substrate for MDR3 (Smith et al., 2000). As in the previous assay, samples of apical media were extracted but quantification of C₆-NBD-PC was performed by fluorescence measurements in a Microplate Fluorimeter.

The result in Fig. 15A shows that C₆-NBD-PC is secreted by BNMG cells. Moreover, the higher specific signal detected (after subtraction of the background WT signal) suggests that the fluorescence-based assay is more sensitive than the TLC-based assay (Fig. 14B) for assessing MDR3 activity. In addition, we also qualitatively analyzed the lipid extract to check for the presence of C₆-NBD-PC-metabolites. Fig. 15B suggests that C₆-NBD-PC is not metabolized by the cells as no other bands than the C₆-NBD-PC band and the PC band appear in the analysis of the apical secreted C₆-NBD-PC by TLC. The PC band results primarily from PC contamination in the albumin used as acceptor, as shown on the right plate in Fig. 15B.

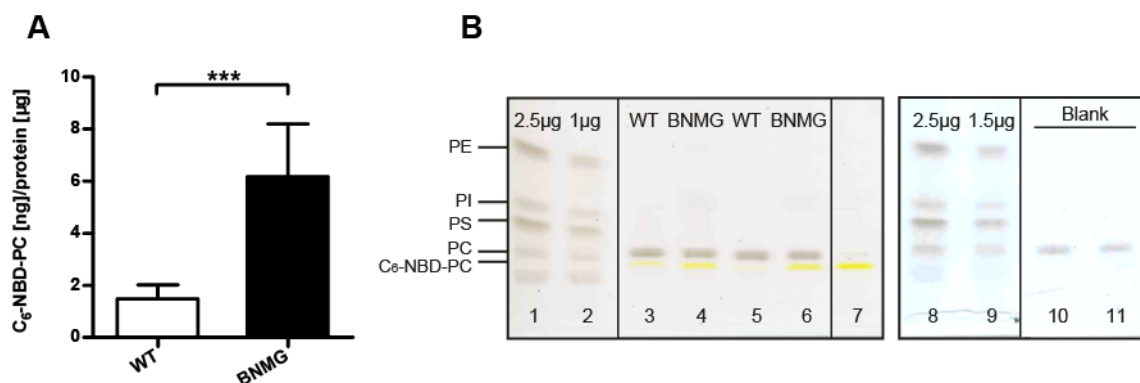


Fig. 15 C₆-NBD-PC secretion by BNMG cells. C₆-NBD-PC was used as substrate and added exclusively to the basolateral compartment in the presence of 1 mM taurocholate basolateral and 50 mg/ml albumin apical. After 24 h the apical medium was collected and extracted. (A) Quantification of the apical secreted C₆-NBD-PC by fluorescence measurements. Results are shown as means of 10 independent experiments \pm SD, ***P < 0.001.

(B) Extracted medium was analyzed by TLC to check for the presence of potential metabolites of C₆-NBD-PC. Lanes 1-2 and 8-9: Phospholipid standards (containing equal amounts of PC: phosphatidylcholine, PS: phosphatidylserine, PI: phosphatidylinositol and PE: phosphatidylethanolamine); Lanes 3-6 Phospholipid and C₆-NBD-PC secreted after 24 h by WT or BNMG cells; Lane 7: medium containing C₆-NBD-PC; Lanes 10-11; apical medium containing albumin.

Moreover, as the release of translocated C₆-NBD-PC into the medium is known to be enhanced by albumin (van Helvoort et al., 1996), and PC secretion has been suggested to occur in a bile salt dependent manner (Morita et al., 2007; Ruetz and Gros, 1995), we tested the effects of these two compounds on C₆-NBD-PC secretion to establish the best conditions to perform drug screening. Fig. 16A shows that supplementation of only taurocholate, to the basolateral compartment did not increase release of C₆-NBD-PC by BNMG cells but supplementation of 50 mg/ml albumin to the

apical compartment dramatically increased C₆-NBD-PC secretion (10-fold) in BNMG cells (Fig. 16B). In addition, Fig. 16B shows that we were able to further increase C₆-NBD-PC secretion by combining these two compounds in our assay. By adding taurocholate to the basolateral compartment and albumin in the apical compartment there was a further increase of secretion of about 29% as compared to supplementation of only albumin. Whether this increase was mediated by a specific interaction between taurocholate and MDR3 or it was only due to an improved solubility of C₆-NBD-PC in the basolateral medium is unclear.

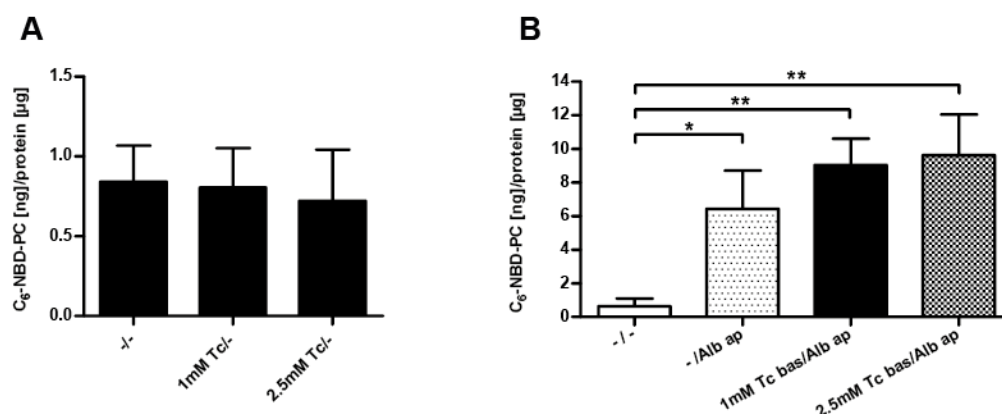


Fig. 16 Effect of taurocholate and albumin on C₆-NBD-PC secretion. (A) The effect of taurocholate on C₆-NBD-PC secretion was assessed by measuring C₆-NBD-PC efflux in BNMG cells in the absence of taurocholate (-/-) and in the presence of 1 or 2.5 mM taurocholate basolateral (Tc bas/-). (B) Effect of taurocholate and albumin on secretion of C₆-NBD-PC was assessed by measuring C₆-NBD-PC efflux in absence of taurocholate and albumin (-/-), in presence of 50 mg/ml albumin in the apical compartment (-/Alb ap), in presence of 1 or 2.5 mM taurocholate in the basolateral compartment and 50 mg/ml albumin in the apical compartment (Tc bas/Alb ap). Results are shown as means \pm SD of 4 independent experiments performed in duplicates or triplicates, * $P < 0.05$, ** $P < 0.01$ versus secretion in absence of acceptor (-/-).

Having set the conditions for our assay, we next tested the functional polarity of MDR3 by comparing transport of C₆-NBD-PC from the basolateral to the apical compartment with the transport from the apical to basolateral compartment. As shown in Fig. 17, in BNMG cells but not in WT cells, the transport of C₆-NBD-PC from the basolateral to the apical compartment was 3 to 4-fold higher than the transport from the apical to the basolateral compartment and no specific apical to basolateral secretion of C₆-NBD-PC could be observed. This result is consistent with the previously shown immunolocalization of MDR3 on the apical membrane of BNMG cells and suggests that in BNMG cells, MDR3 was functionally active and mediated vectorial phosphatidylcholine transport into the apical compartment.

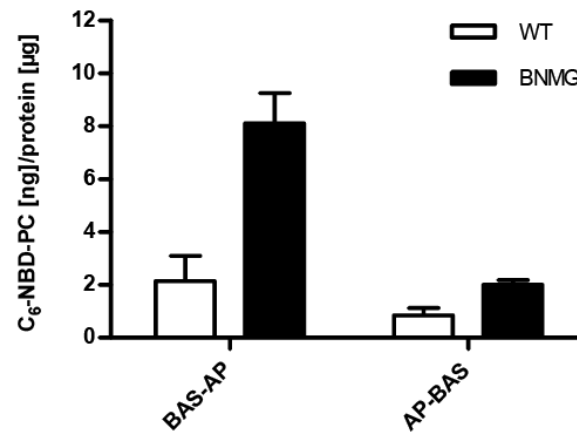


Fig. 17 Vectorial transport of C₆-NBD-PC in BNMG cells. Transport of C₆-NBD-PC from basolateral to apical and from apical to basolateral was assessed in WT and BNMG cells using the Transwell[®] system. Cell monolayers were incubated with 1 mM taurocholate and C₆-NBD-PC as substrate either in the basolateral or in the apical compartment and with 50 mg/ml albumin in the corresponding acceptor compartment. After 24 h the medium of the acceptor compartment was collected and processed to quantify the secreted C₆-NBD-PC. Results are shown as means from 2 independent experiments performed in duplicates \pm SD.

4.1.7 Expression of endogenous MDRs in LLC-PK₁ cells

The minimal amount of C₆-NBD-PC observed in the apical medium of WT cells in Fig. 16A and Fig. 17 could be MDR1-mediated, as previous experiments have shown that C₆-NBD-PC can be transported also by MDR1 (Smith et al., 2000). To rule out a significant role of this ABC-transporter in apical C₆-NBD-PC secretion in our model cell line, we analyzed our cell lines for the expression of endogenous pMDR1. Using the antibody C219, reported to cross-react with porcine MDR1 (Crivellato et al., 1999) and known to recognize a common epitope on MDR1 and MDR3 (van Den Elsen et al., 1999), practically no signal was observed in membranes isolated from WT cells, even after treatment of cells with sodium butyrate (Fig. 18B), but the expected MDR3 signal (140 kDa) was detected in membranes of BNMG cells. Membranes isolated from Caco-2 cells were used as MDR1 positive control (Hosoya et al., 1996; Uchida et al., 2015) and displayed a band at the predicted MDR1 size (170 kDa). Fig. 18A shows the corresponding blot with an antibody against MDR3, known to not cross-react with MDR1 (Scheffer et al., 2000) and hence without signal in WT and Caco-2 cells. These data indicate that expression of pMDR1 in our cell lines should have been minimal. Hence, measuring C₆-NBD-PC secretion into the apical compartment should reflect PC transport by MDR3.

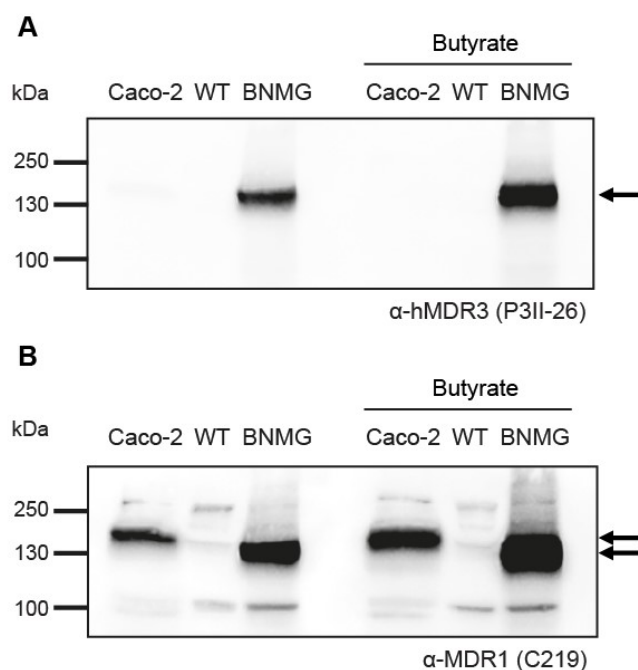


Fig. 18 Western blot analysis of MDR3 and MDR1. Total membranes of Caco-2 cells, and LLC-PK₁ WT cells and BNMG cells with/without overnight pretreatment with 5 mM sodium butyrate were isolated and analyzed for MDR1 and MDR3 expression by Western blotting. Lanes were loaded with 100 µg membrane proteins. (A) LLC-PK₁ WT cells and BNMG cells were probed for MDR3 using a specific MDR3 antibody. (B) LLC-PK₁ WT cells and BNMG cells with/without overnight pretreatment with 5 mM sodium butyrate were probed for endogenous MDR1 using an antibody recognizing both MDR1 and MDR3 (C219). Caco-2 cells were loaded as MDR1 control.

4.1.8 Validation of model cell line for studying drug interactions with MDR3

Smith and coworkers (Smith et al., 2000) demonstrated that MDR3 is inhibited by verapamil and by PSC833. Therefore, we used these compounds to functionally validate our model cell line. Fig. 19A shows that MDR3-mediated secretion of C₆-NBD-PC was reduced by 40% by verapamil and by 52% by PSC833. In addition, Fig. 19B shows that MDR3 protein levels were stable in BNMG cells treated with verapamil or PSC833 suggesting that the reduction of C₆-NBD-PC secretion was not due to a reduction of MDR3 expression but rather to inhibition of MDR3. Hence, BNMG cells are suitable for investigating the interaction of drugs with MDR3.

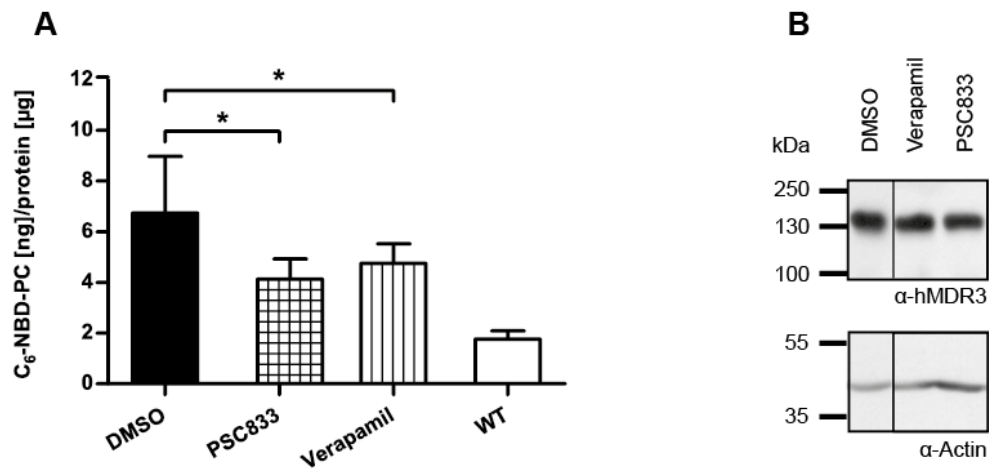


Fig. 19 Control for inhibition of MDR3-mediated C₆-NBD-PC transport. (A) Apical C₆-NBD-PC secretion, in the presence of 1 mM taurocholate basolateral and 50 mg/ml albumin apical, was assessed in cells treated 24 h with 10 μM verapamil or 4 μM PSC833. Results are shown as means ± SD of 5 independent experiments performed in triplicates, *P < 0.05 versus DMSO. (B) Western blotting of Transwell® monolayers of BNMG cells previously treated 24 h with 10 μM verapamil and 4 μM PSC833 was performed. Lanes were loaded with 70 μg cell lysate protein.

4.2 Application of the established *in vitro* model for testing drugs interactions with hepatobiliary transporters

In contrast to BSEP, where inhibition by drugs is a well-established cause of acquired cholestasis (Bhamidimarri and Schiff, 2013; Stieger, 2010), the role of MDR3 in DILI is not clear and reports of DILI as a consequence of MDR3 inhibition by drugs are very rare. Therefore, in the second part of this project we were interested in assessing the effect of selected drug candidates on MDR3 activity using the established *in vitro* system in conjunction with the previously described fluorescence-based assay. Based on the literature, interesting drug candidates were selected as potential inhibitors or “stimulators” of MDR3.

4.2.1 Interaction of antifungals with MDR3

In patients, a cholestatic pattern of DILI is characterized by a predominantly elevated alkaline phosphatase (AP) (Aithal et al., 2011). In addition, inherited impairment of MDR3 (e.g. PFIC3) leads to a typical and specific (as this is not the case in impairment of BSEP e.g. PFIC2) increase of serum γ -glutamyltranspeptidase (γ -GTP) (Jacquemin, 2012). Therefore, drugs reported to elicit mainly an elevation of AP and/or γ -GTP were selected to be studied in our *in vitro* model system. Several patients on itraconazole therapy have been reported to have increased serum ALT (alanine aminotransferase), AP, γ -GTP and bilirubin suggesting DILI with a mixed hepatocellular and cholestatic pattern (Lavrijsen et al., 1992; Yoshikado et al., 2011). Moreover, a study reported MDR3 inhibition by itraconazole (Yoshikado et al., 2011). Therefore, we started by testing the effect of itraconazole. As shown in Fig. 20 we could reproduce the inhibitory effect of itraconazole on MDR3 also in our *in vitro* model system.

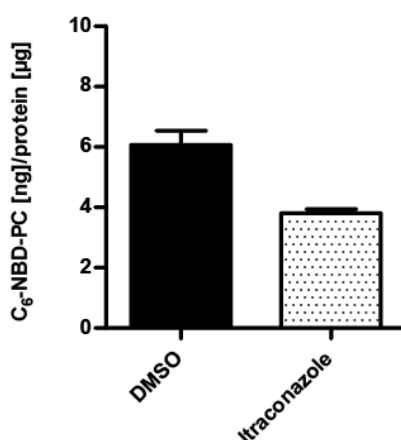


Fig. 20 Effect of itraconazole on MDR3-mediated C₆-NBD-PC secretion. BNMG monolayers were treated 24 h with 10 μ M itraconazole or DMSO (as control) in the presence of 1 mM taurocholate basolateral and 50 mg/ml albumin in the apical compartment and C₆-NBD-PC secretion in the apical compartment was assessed. Results are shown as means \pm SD of 2 independent experiments performed in triplicates.

This result was further validated by measuring the effect of itraconazole on PC secretion. Cells were pre-incubated in radioactively labelled ^{14}C -choline as precursor of PC. Subsequently, cells were treated with itraconazole and secretion of ^{14}C -PC in the apical compartment was analyzed by TLC. As shown in Fig. 21C, itraconazole leads to a remarkable decrease of secreted ^{14}C -PC, confirming the previous results showing inhibition of MDR3-mediated C_6 -NBD-PC secretion.

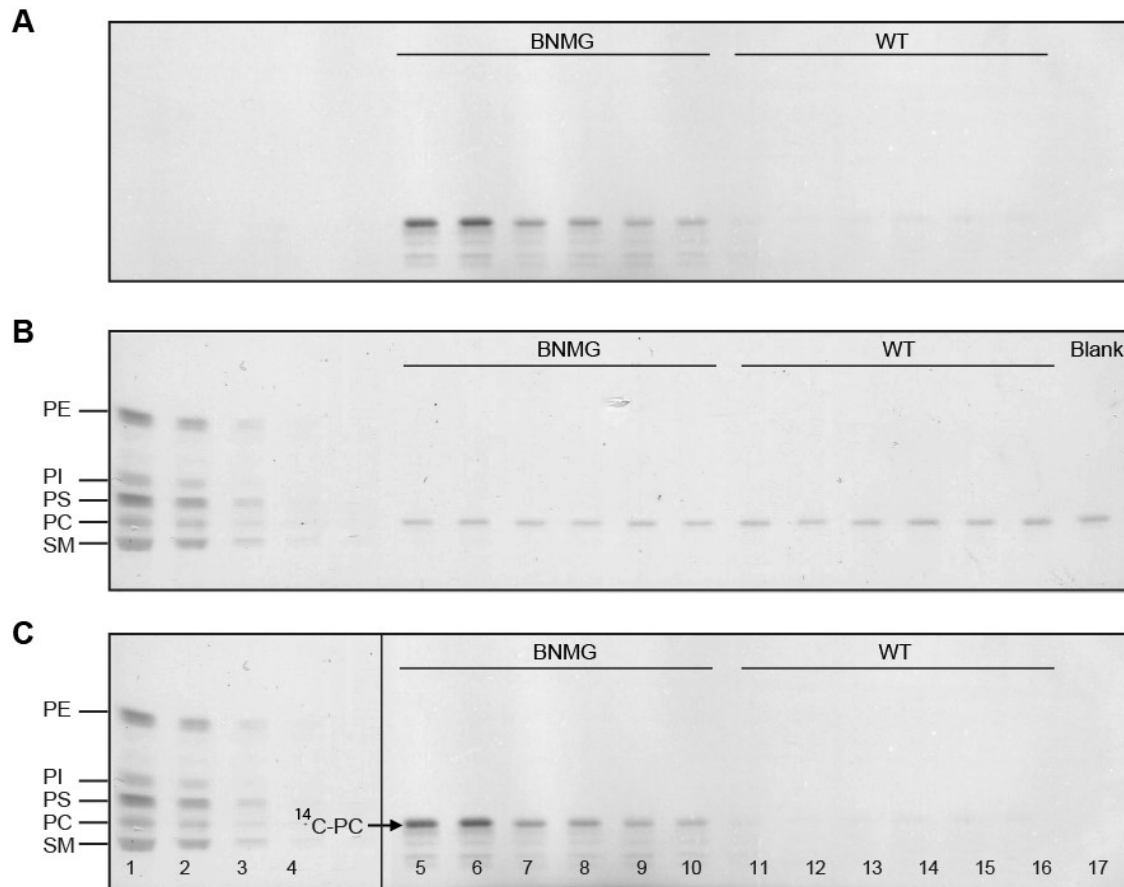


Fig. 21 Effect of itraconazole on MDR3-mediated ^{14}C -PC secretion. Cell monolayers were pre-incubated in MEM medium containing ^{14}C -choline and subsequently treated overnight with itraconazole in the presence of 1 mM taurocholate basolateral and 50 mg/ml albumin apical. Apical media were extracted and separated by TLC to assess the secreted ^{14}C -PC. (A) Film exposed to HPLC plate detecting ^{14}C -choline radiolabeled lipids. (B) Stained HPTLC plate showing total phospholipids (C) Composite picture of right side of (A) and left side of (B) to compare the migration of the radiolabeled peptides to the standards (not radioactively labelled). Lanes 1-4: phospholipid standards (SM: sphingomyelin, PS: phosphatidylserine, PC: phosphatidylcholine, PI: phosphatidylinositol, PE: phosphatidylethanolamine); Lanes 5-6: BNMG cells treated with DMSO (control); Lanes 7-8: BNMG cells treated with 2 μM itraconazole; Lanes 9-10: BNMG cells treated with 10 μM itraconazole; Lanes 11-12: WT cells treated with DMSO (control); Lanes 13-14: WT cells treated with 2 μM itraconazole; Lanes 15-16: WT cells treated with 10 μM itraconazole; Lane 17: albumin containing medium.

Based on clinical studies on DILI, many azoles have been associated with increased hepatic enzymes and hepatotoxicity (Gearhart, 1994; Somchit et al., 2004; Yoshikado et al., 2011). Therefore, we next

studied the impact of different azoles on MDR3 function to find out whether as for itraconazole, MDR3 inhibition may be implicated in the pathologic mechanism leading to DILI. Fig. 22A reveals that in addition to itraconazole, also ketoconazole and posaconazole inhibited MDR3 with decreasing potency: posaconazole > itraconazole > ketoconazole. Fluconazole and voriconazole showed no inhibition of MDR3. Next, we determined the IC_{50} value of the inhibition of C₆-NBD-PC secretion by posaconazole, which was found to be 4.2 μ M (Fig. 22B) suggesting that posaconazole is a specific MDR3 inhibitor. Attempts to determine the IC_{50} value of itraconazole failed due to the cytotoxicity of this drug at higher concentrations.

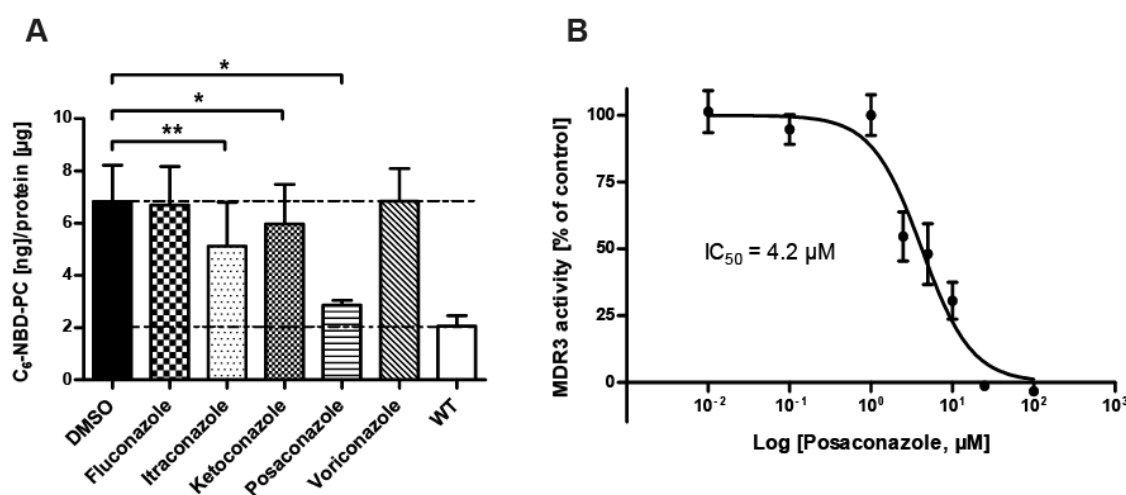


Fig. 22 Interaction of azoles with MDR3. (A) BNMG monolayers were treated 24 h with 10 μ M azoles (in the presence of 1 mM taurocholate basolateral and 50 mg/ml albumin in the apical compartment) and C₆-NBD-PC secretion was assessed. Results are shown as means \pm SD of 4 independent experiments performed in duplicates to quadruplicates, * P < 0.05, ** P < 0.01. (B) A dose-response curve for MDR3 inhibition by posaconazole was generated by assessing C₆-NBD-PC secretion in BNMG cells in presence of different concentrations of posaconazole and subtracting the background (C₆-NBD-PC secretion in treated WT). The percentual MDR3 activity was plotted against the logarithm of the concentration of inhibitor/posaconazole to determine the IC_{50} . An IC_{50} of 4.2 μ M (95% confidence interval, CI 3.1-5.8) was calculated using GraphPad Prism. Data represent the mean \pm SD of 2 independent experiments performed in duplicates.

To exclude an interference of the azoles with MDR3 protein levels, the expression of MDR3 in azoles treated BNMG monolayers was compared to control BNMG cells (treated with DMSO) by Western Blotting. Fig. 23A shows that itraconazole and posaconazole induced specifically MDR3 protein levels, while the other azoles had no effect on MDR3 protein. Hence, we could rule out that reduced MDR3 protein expression was the reason for the inhibition of transport. Fig. 24A show that the increase in MDR3 protein levels upon itraconazole treatment was time-dependent and hence likely caused by a cellular response to itraconazole. In contrast, BSEP and NTCP expression levels remained unchanged after treating BNMG cells with different azoles. In addition, quantification of

the induction of MDR3 protein levels by itraconazole over 48 h was performed using the blots shown in Fig. 23B and Fig. 24A and confirmed these results (Fig. 24B).

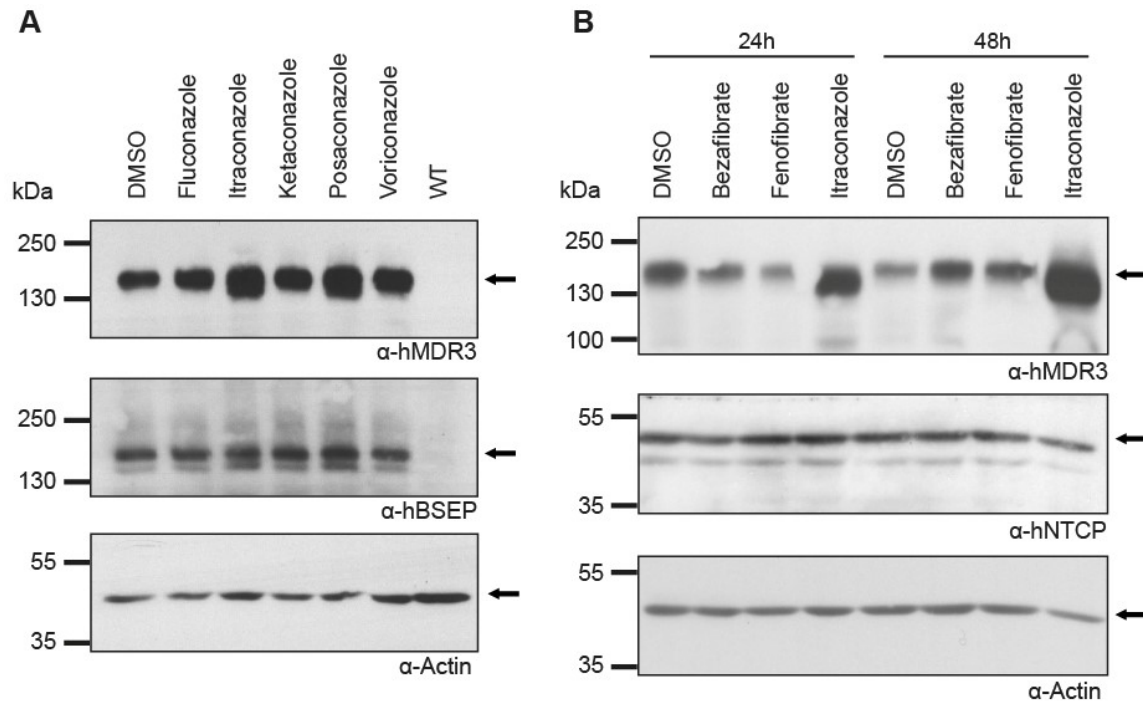


Fig. 23 Effect of azoles on MDR3, BSEP and NTCP protein levels. (A) Western blotting of Transwell® monolayers of BNMG cells previously treated 24 h with 10 μ M azoles was performed. Lanes were loaded with 70 μ g cell lysate protein. (B) Western blotting of cell lysates of BNMG cells cultured on 10 cm dishes and treated 24 and 48 h with 10 μ M itraconazole, 200 μ M bezafibrate or fenofibrate (PPR α agonists). Lanes were loaded with 70 μ g cell lysate protein. On both blots, β -actin was immunoblotted as loading control.

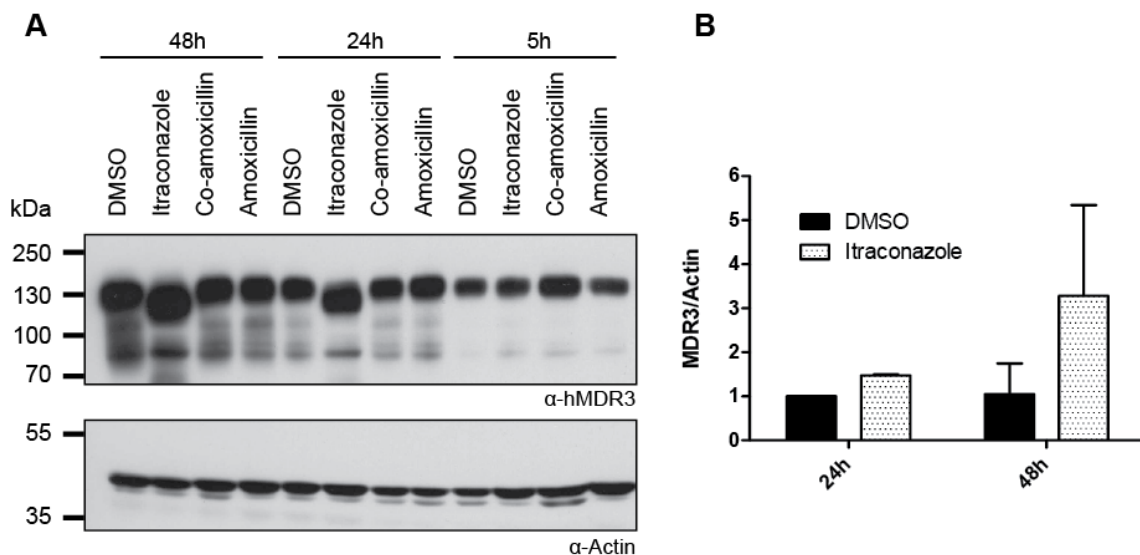


Fig. 24 Time-dependent induction of MDR3 protein levels by itraconazole. (A) Western blotting of cell lysates of BNMG cells cultured on 10 cm dishes and treated 5, 24 and 48 h with 10 μ M itraconazole, 100/300 μ M co-amoxicillin and 300 μ M amoxicillin was performed. (B) Quantification of the effect of itraconazole on MDR3 protein levels over 48 h. Results were normalized to DMSO and are shown as mean \pm SD of 2 independent experiments.

To rule out that the increase of MDR3 protein levels were induced at the transcriptional level we measured mRNA levels in BNMG cells after treatment with itraconazole. Results in Fig. 25 indicate that as expected MDR3 regulation on the transcriptional level is very unlikely to be involved in the mechanism leading to this increase. In fact, this is unlikely as the expression of the stably transfected MDR3 is under a constitutive CMV promoter.

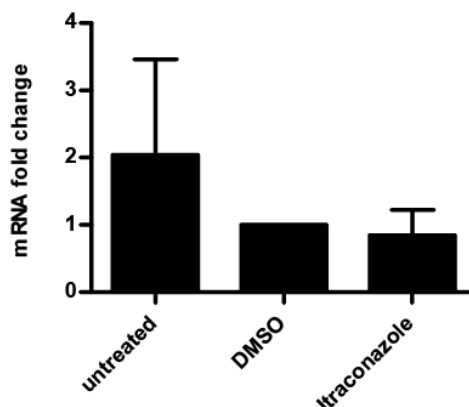


Fig. 25 Effect of itraconazole on MDR3 mRNA. BNMG cells grown on dishes were treated 24 h with itraconazole and mRNA levels were assessed by Quantitative Real-Time qRT-PCR. Results are shown as means of 2 independent experiments performed in triplicates \pm SD.

4.2.2 Interaction of antifungals with bile salt transporters

Since inhibition of BSEP contributes to DILI (Pauli-Magnus and Meier, 2006), we assessed the impact of azoles also on taurocholate transport in BNMG cells. Fig. 26A shows that ketoconazole, itraconazole, posaconazole and PSC833 inhibited apical secretion of taurocholate. Furthermore, analysis of intracellularly retained taurocholate showed an accumulation in conditions where apical taurocholate secretion was impaired suggesting that the reduced transcellular bile salt flux measured after treatment of monolayers with itraconazole, ketoconazole, and posaconazole resulted mainly as a consequence of BSEP inhibition and rather not of NTCP inhibition (Fig. 26B). In contrast, PSC833 which also potently inhibited taurocholate transport to the apical compartment did not show a parallel increase of the intracellular taurocholate levels as the above mentioned azoles. This indicated that in the case of PSC833, inhibition of NTCP may have contributed to the inhibition of the transcellular bile salt flux.

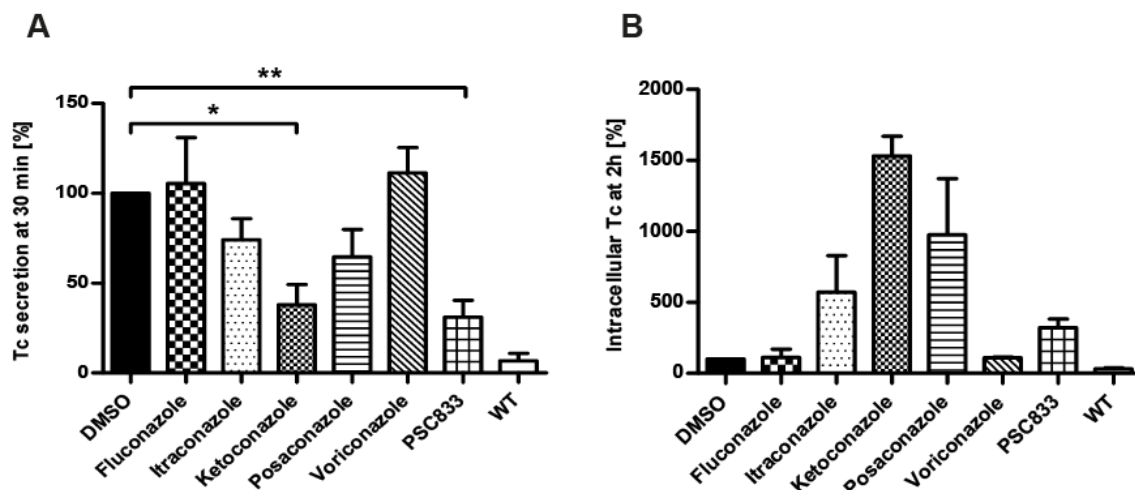


Fig. 26 Interaction of azoles with transcellular taurocholate transport. Monolayers were treated with 10 μ M azoles or 4 μ M PSC833 for 24 h and transport experiments were started by adding 10 μ M taurocholate to the basolateral compartment. (A) The effect of azoles on taurocholate transport was assessed by measuring the amount of taurocholate transported to the apical compartment after 30 min. The total taurocholate secreted in the apical compartment was normalized to the total amount of protein per monolayer. Drugs-treated monolayers were normalized to control monolayers treated with DMSO. Results are shown as means \pm SD of 3 independent experiments performed in duplicates, * $P < 0.05$ versus DMSO. (B) The intracellular accumulation of taurocholate was assessed as indicator for BSEP inhibition. After 2 h the monolayers were washed once with PBS and solubilized in 1% Triton X-100 and the intracellular retained taurocholate was assessed. Results were normalized to the protein's amount. Drug-treated monolayers were normalized to DMSO-treated monolayers. Results are shown as means \pm SD of 2 independent experiments performed in duplicates.

4.2.3 Interaction of additional hepatotoxic drugs with MDR3

In addition to the antifungal-azoles, several other drugs belonging to different pharmacological classes have been associated with DILI. Therefore, additional selected drug candidates were tested for a potential inhibition of MDR3 activity.

The antibiotic amoxicillin–clavulanate (co-amoxicillin) has been found to be the most common drug associated with DILI (Bjornsson, 2015; Leise et al., 2014). Bupropion and nefadozone are antidepressants drugs, both associated with DILI (Park and Ishino, 2013). Bupropion-induced hepatotoxicity is not well defined as in most case reports patients were concomitantly taking other drugs (Park and Ishino, 2013). In contrast, although the mechanism remains unknown, liver toxicity associated with nefadozone is well-established and in 2001 the FDA issued a “black box” warning of the potential irreversible liver failure induced by this drug (Park and Ishino, 2013). In the literature, several cases of drug-induced cholestasis by the antipsychotic drug chlorpromazine are reported (Derby et al., 1993). Olanzapine is an atypical antipsychotic drug associated to DILI (Devarbhavi et al., 2010) and which has been shown to inhibit MDR1 (Wang et al., 2006). As MDR1 is the closest homolog to MDR3 (van der Blik et al., 1988), it is conceivable that olanzapine may also modulate

MDR3 by recognizing common conserved domains (Morita et al., 2007). Troglitazone was a drug used for treatment of type II diabetes and was withdrawn from the market because of the numerous reports of liver failure. Multiple mechanism for troglitazone hepatotoxicity such as mitochondrial injury and BSEP inhibition are discussed (Smith, 2003). Cyclosporin A is a well-established BSEP inhibitor (Stieger, 2010) and has a very similar structure to PSC833. Finally, octreotide is used to treat acromegaly, thyroid stimulating hormone secreting pituitary adenomas and neuroendocrine tumors and about half of patients receiving octreotide have gallbladder stones (Dowling et al., 1992). The results in Fig. 27A suggest that with the exception of cyclosporine A, none of the tested drugs inhibited MDR3.

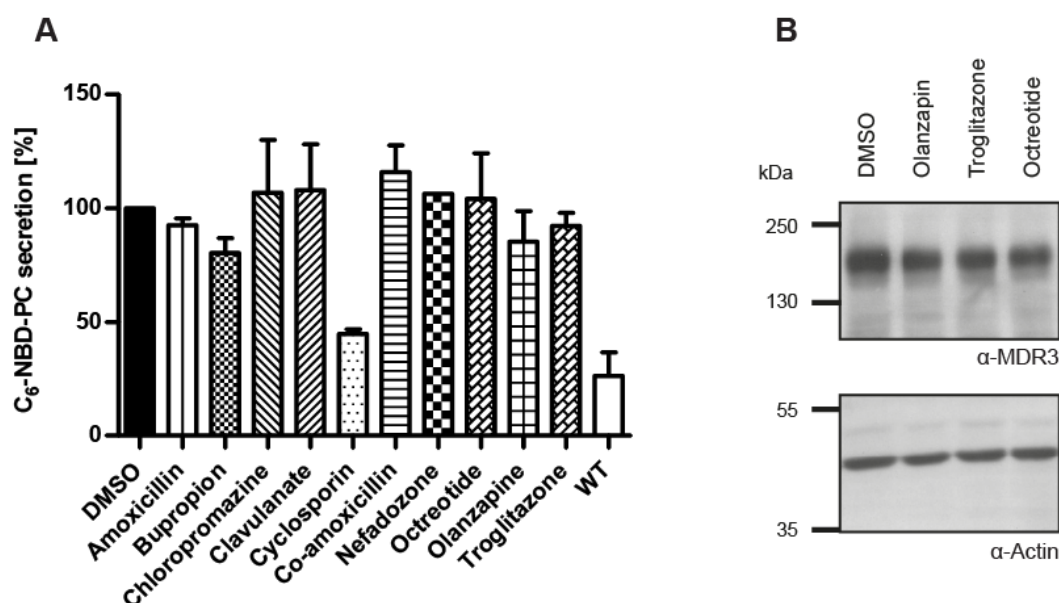


Fig. 27 Effect of hepatotoxic drugs on MDR3 activity. Cell monolayers were treated 24 h with 300 μ M amoxicillin, 10 μ M bupropion, 5 μ M chlorpromazine, 100 μ M clavulanate, 1 μ M cyclosporin A, 100/300 μ M co-amoxicillin (amoxicillin and clavulanate), 10 μ M nefadozone, 25 μ M octreotide, 10 μ M olanzapine, and 10 μ M troglitazone to assess their effect on MDR3-mediated C₆-NBD-PC secretion. Results are shown as means \pm SD of 3 or 2 independent experiments performed in duplicates or triplicates. (B) Western blotting of the corresponding Transwell monolayers of BNMG cells previously treated 24 h with the drugs was performed. Lanes were loaded with 70 μ g cell lysate protein.

4.2.4 Interaction of cholagogues with MDR3

In addition to drugs associated with hepatotoxicity and thus potential inhibitors of hepatobiliary transporters, cynarin, boldine and luteolin were tested for a potential “stimulation” of MDR3 activity, which would be beneficial to the bile ducts. These substances are the active ingredients of herbal “cholagogues”. In traditional herbal medicine “cholagogues” are used against gastrointestinal disorders to stimulate bile flow (choleresis). A well-known, old cholagogue used against gastrointestinal and liver disorders is artichoke leaf extract (*Cynara scolymus* L.). Artichoke leaf extract was reported to stimulate biliary secretion in humans (Kirchhoff et al., 1994; Matuschowski et al., 1997) and to have lipid lowering properties (Adzet et al., 1987; Kraft, 1997; Wegener and Fintelmann, 1999). The active ingredients in artichoke leaf extract are cynarin and luteolin (Salem et al., 2015). Similarly, boldo leaf extract (*Peumus boldus* Mol.) with its active ingredient boldine is used against liver ailments in South America (Speisky and Cassels, 1994) and reportedly stimulates choleresis (O'Brien et al., 2006; Zagorova et al., 2015). Fig. 28A and Fig. 28B show that in our cell-based model, these substances had no effect on MDR3 activity as well as on MDR3 protein levels.

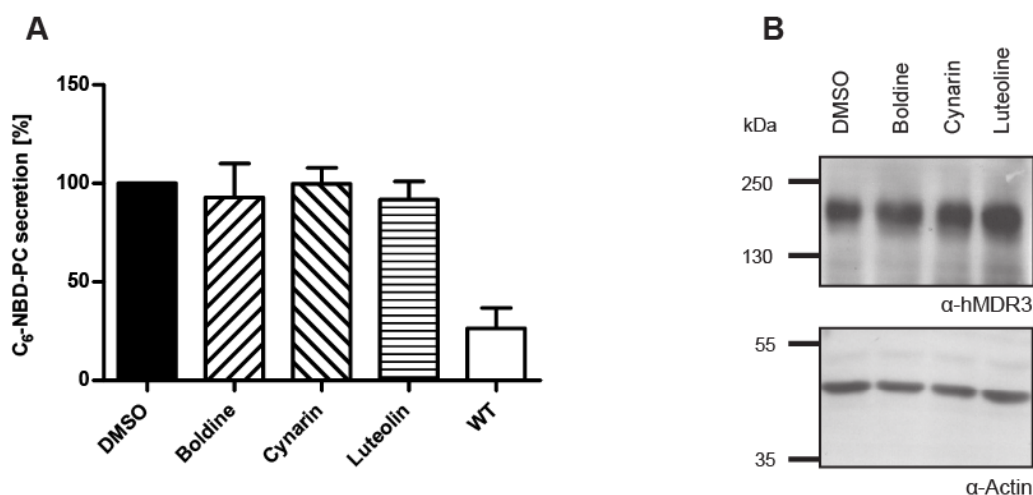


Fig. 28 Effect of cholagogues on MDR3 activity. (A) Cell monolayers were treated 24 h with 10 μ M luteolin, 10 μ M boldine and 100 μ M cynarin and their effect on C₆-NBD-PC secretion was assessed. Results are shown as means \pm SD of 3 independent experiments performed in duplicates or triplicates. (B) Western blotting of corresponding Transwell[®] monolayers of BNMG cells previously treated 24 h cholagogues was performed. Lanes were loaded with 70 μ g cell lysate protein.

4.2.5 Interaction of fibrates with MDR3

Fibrates have been reported to stimulate phosphatidylcholine secretion by a transactivation of MDR3 gene transcription (Ghonem et al., 2014). However, in the literature also other mechanisms leading to increased phosphatidylcholine secretion such as redistribution of MDR3 in bile canaliculi (Shoda et al., 2004; Shoda et al., 2007) have been described. Therefore, we were interested to test the effect of these drugs in our *in vitro* model. As in our BNMG cells MDR3 is constitutively expressed, MDR3 protein levels remained stable upon treatment with fibrates (Fig. 29B). Due to the higher variability of the results, alterations of C₆-NBD-PC secretion upon fibrates treatment were not statistically significant (Fig. 29A).

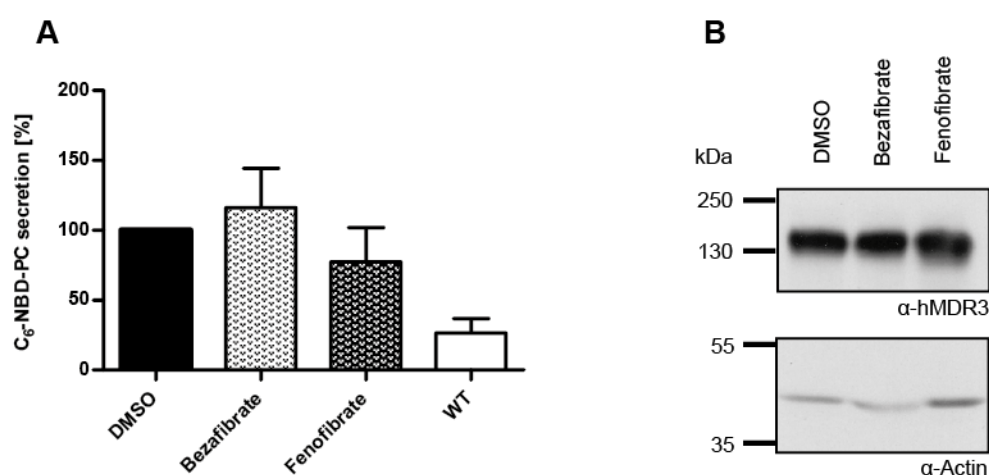


Fig. 29 Effect of fibrates on MDR3 activity. (A) Cell monolayers were treated 24 h with 200 μ M bezafibrate or fenofibrate their effect on C₆-NBD-PC secretion was assessed. Results are shown as means \pm SD of 5 independent experiments performed in duplicates or triplicates. (B) Western blotting of the corresponding Transwell[®] monolayers of BNMG cells previously treated 24 h fibrates was performed. Lanes were loaded with 70 μ g cell lysate protein.

5 Discussion

The established cell-based model system for canalicular bile salts and phosphatidylcholine secretion is functional and was used to assess the effect of several hepatotoxic drugs as well as potential hepatoprotective compounds on canalicular secretion. Of the several drugs tested, the azoles posaconazole, itraconazole and ketoconazole, used to treat antifungal infections, inhibited both BSEP and MDR3 to varying extents. This may explain one of the mechanisms of drug-induced liver injury of this class of drugs. Furthermore, interestingly posaconazole and itraconazole, in parallel to inhibiting MDR3, induced MDR3 protein levels. Other compounds did not alter MDR3 activity, suggesting that their hepatotoxic or hepatoprotective effects are not caused by direct interactions with MDR3.

5.1 Characterization of a model cell line for biliary lipid secretion

5.1.1 Overview of the established *in vitro* model

In the first part of this project, we constructed and characterized an *in vitro* model for canalicular phospholipid secretion consisting of stably transfected LLC-PK₁ cells, expressing functional NTCP, BSEP and MDR3 that are cultured to tight monolayers in the Transwell® system (Fig. 30). This model system allows testing for the interaction of drugs with canalicular lipid and bile salt secretion. The established model cell system displays the following advantages as compared to other *in vitro* models for studying biliary phospholipid and/or bile salts secretion: 1) direct experimental access to the basolateral and apical plasma membranes, which is precluded in hepatocytes. This allows, if desired, the supplementation of substrates to both compartments and the preloading of the apical compartment with acceptor proteins to ensure solubility of the translocated lipids; 2) formation of a tight epithelium with the possibility to generate transcellular fluxes for bile salts and lipids and hence the reconstitution of trans-hepatocellular fluxes of cholephilic compounds; 3) the possibility to simultaneously assess the impact of drugs on BSEP-mediated bile salts and MDR3-mediated lipid secretion; 4) the availability of a control LLC-PK₁ WT cell line without the added human transporters (which is not available for. e.g. in hepatocytes-based models) and a high signal-to-noise ratio, allowing a clean interpretation of the data and no bias in calculating specific transport; 5) a low expression of drug metabolizing enzymes (Gonzalez and Tarloff, 2004) which can be helpful to delineate the individual contribution of drug and metabolites to transporter inhibition by testing them separately.

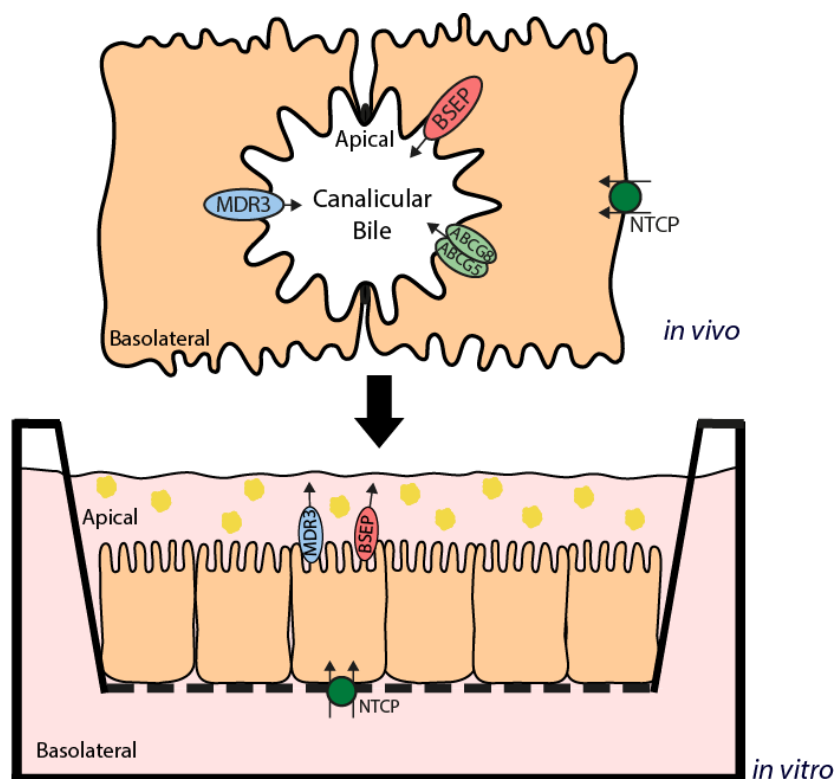


Fig. 30 Overview of the established *in vitro* model system for canalicular lipid secretion. The upper part of the picture represents the *in vivo* situation in human hepatocytes. The lower part of the picture depicts our established *in vitro* model constituted of stably transfected and polarized LLC-PK₁ cells grown on Transwell® filters. The cells express NTCP on the basolateral domain and MDR3 and BSEP on the apical domain.

5.1.2 Analysis of expression and localization of the transporters

Western blot analysis of cell lysates and immunohistochemical analysis of cell monolayers demonstrated expression and polarized localization of human NTCP, BSEP and MDR3 in the transfected LLC-PK₁ cell line named BNMG. Immunolocalization of the transporters was performed with cells cultured on Transwell® filters using the same conditions as for the functional transport assays. Therefore, the recorded pictures are representative for the cells used for the functional assays. These data demonstrated that NTCP was expressed on the basolateral membrane, whereas BSEP and MDR3 were expressed in the apical domain of LLC-PK₁ suggesting that the BNMG cells mimics, at least qualitatively, human hepatocytes (Buschman et al., 1992; Stieger, 2011). Absolute quantitative data of the transporter protein levels and comparison with human hepatocytes would help to assess the suitability of the developed model for *in vitro* to *in vivo* extrapolations. There have been studies showing missorting of basolateral proteins in LLC-PK₁ cells due to a lack of AP-1B expression (Cao et al., 2012). AP-1B is a clathrin-associated adaptor protein expressed in various polarized epithelial cell lines including MDCK and Caco-2 cells (Ohno et al., 1999). The fact, that NTCP was sorted correctly to the basolateral domain in our BNMG cells suggests that the basolateral sorting of NTCP does not require AP-1B but another sorting mechanism.

5.1.3 The bile salt transporters, NTCP and BSEP

Functional polarity of the bile salt transporters was assessed by comparing the transcellular transport and the intracellular accumulation of the radioactively labelled bile salt taurocholate after supplementing it either to the basolateral or to the apical compartment. The bile salt transport rate and the intracellularly accumulated bile salt were remarkably higher when taurocholate was added to the basolateral compartment of BNMG cells suggesting that NTCP and BSEP mediated a vectorial transport of bile salts through the basolateral and apical membrane and hence mimicked hepatocellular bile salt flux.

Of notice, by measuring the intracellularly accumulated bile salt, it was possible to get insights on whether a decreased bile salt flux was due to BSEP or NTCP inhibition by a drug.

5.1.4 The cholesterol transporter, ABCG5/G8

ABCG5/G8-mediated cholesterol secretion has been suggested to require bile salts (Vrins et al., 2007). Therefore, cells were treated with different concentrations of taurocholate either in the basolateral, in the apical or in both compartments to stimulate secretion of cholesterol. However, as with concentration of taurocholate exceeding 2 mM we observed an increased LDH release and a leakage in our monolayers (assessed by Lucifer yellow integrity test), both indicative of impacted cell viability, we chose to perform our experiments with 1 mM taurocholate. Both transport of radioactively labelled cholesterol as well as endogenous cholesterol secretion to the apical compartment were measured. For both substrates, supplementation of albumin or apoA1 as acceptors in the apical compartment resulted in an at least two-fold increase of cholesterol secretion. However, the signal in the cells transfected with ABCG5/G8 was not significantly higher than WT cells. Hence cholesterol secretion in our BNMG model cell line was not an ABCG5/G8-mediated process. This raises the question whether the ABCG5/G8 heterodimer was functional and/or expressed at sufficient level. In order to assess the expression levels of ABCG5/G8 we analyzed the total cell lysate of BNMG cells and could detect a weak specific signal for ABCG8 in BNMG cells. However, for ABCG5 we had to use extracted membrane fractions in order to see a specific signal. Moreover, when comparing the signal of ABCG5 in BNMG cells to Caco-2 cells, which endogenously express ABCG5 (Grenier et al., 2012), the signal in BNMG cells is much lower. Hence, these data suggest that expression levels of ABCG5/G8 could be too low to detect a phenotype. Alternatively, the expressed ABCG5/G8 might be non-functional possibly because they do not heterodimerize or another unknown co-factor, needed for efficient ABCG5/G8 mediated cholesterol transport, could be missing in BNMG cells. Finally, taurocholate may be able in our model system to unspecifically extract cholesterol from the plasma membrane, which in turn could mask a potential contribution of ABCG5/G8 to cholesterol release.

5.1.5 The phosphatidylcholine transporter, MDR3

The predominant phospholipid in human bile is phosphatidylcholine (Alvaro et al., 1986; Phillips, 1960). Phosphatidylcholine secretion into bile is mediated by MDR3, a specific PC translocase. Chemical analysis of the phospholipids secreted into the apical compartment, as well as transport experiments with C₆-NBD-PC, showed a significantly higher PC and C₆-NBD-PC secretion in the apical compartment of BNMG cells (than in WT cells) suggesting that MDR3 was functionally active in BNMG cells. Furthermore, since an increased secretion of C₆-NBD-PC was also measurable in the absence of taurocholate (in Fig. 16B), it is unlikely that the increased C₆-NBD-PC secretion was only due to an indirect effect of the bile salt flux mediated by BSEP and NTCP in BNMG cells. Further control experiments to exclude unspecific effects leading to an increased MDR3-independent phospholipid secretion could include the analysis of phospholipid secretion in a BNG cell line expressing BSEP, NTCP and ABCG5/G8 but not MDR3 or the analysis of phospholipid secretion before and after the suppression of MDR3 with siRNA.

As compared to the quantification of endogenous PC secretion, a higher specific signal was detected by the quantification of C₆-NBD-PC secretion. This may be due to the following reasons: 1) contamination of albumin with PC were variable and generated a high background signal that interfered with the signal of the PC secreted by the cells. This may have been a source of error in PC quantification. Of notice is that analysis of different albumin lots and batches displayed different, highly variable amounts and quality of lipids. Companies which sell fatty acid free albumin specify only the maximal possible lipid content but do not define the exact amount or the types of lipid contained; 2) Moreover, as compared to quantification of C₆-NBD-PC, which is based on fluorescence measurements of apical medium extracts, quantification of endogenous PC by TLC involves additional technical steps such as loading, developing and staining the TLC plates which may also contribute to the increase of the variability; 3) Finally, secretion of PC relies on the endogenous pool of PC, which relies itself on the rate of PC biosynthesis. Biosynthesis of PC in LLC-PK₁ cells could be the limiting step of endogenous PC secretion. Hence, as the C₆-NBD-PC based assay was not only more sensitive but also faster and had a better reproducibility, we decided to use this assay also for testing drug interactions with MDR3. In the literature only a few different approaches to study drug interactions with MDR3 have been described: van Helvoort A and coworkers used C₆-NBD-PA as C₆-NBD-PC precursor (van Helvoort et al., 1996), Yoshikado et al. used ¹⁴C-choline as precursor of phosphatidylcholine (Yoshikado et al., 2011) and very recently He et. al. published a method using choline-d₉ as precursor and assessed deuterium (d₉)-labelled PC secretion by LC-MS/MS (He et al., 2015). As compared to these assays our C₆-NBD-PC-based assay display the following main differences: 1) C₆-NBD-PC is not a precursor molecule and therefore results are not influenced by alterations in the biosynthetic pathway as compared to the previous methods; 2) Fluorescence is a sensitive, easy and cheap method to quantify lipid as compared to

radioactivity measurements or LC-MS/MS based measurements; 3) C₆-NBD-PC is a surrogate substrate possessing a different chemical structure than PC and therefore a different K_M for MDR3. The different chemical structure of C₆-NBD-PC could also explain why, in contrast to what has been previously reported for PC (Morita et al., 2007), supplementation of only taurocholate did not dramatically increase C₆-NBD-PC secretion (Fig. 16A). Although, another possible explanation for this could be that the amounts of taurocholate that from the basolateral compartment reached the apical compartment were too low to enhance C₆-NBD-PC secretion. To clarify this, additional experiments, involving also supplementation of taurocholate directly to the apical compartment as well as the determination of the apical concentration of taurocholate after its supplementation to the basolateral compartment, would be required. As we knew that treating cells with taurocholate concentrations above 2 mM increases LDH release, a sign of cell necrosis, we decided to keep albumin as main acceptor in the apical compartment to avoid unspecific effects of taurocholate which may alter apical membrane permeability. Therefore, we used throughout the work only 1 mM taurocholate in the basolateral compartment. Interestingly, in both assays (with PC and C₆-NBD-PC) used to assess MDR3 function, supplementation of 50 mg/ml albumin in the apical compartment dramatically increased phospholipids released in the apical compartment in BNMG cells. Albumin is a much larger molecule than taurocholate. Therefore, these results, partially contradicts the model proposed by Morita and coworkers, according to which bile salts (e.g. taurocholate) may bind PC still in cavity or on the surface of MDR3 (Morita et al., 2007) and support rather the hypothesis that the phospholipid acceptor, bile salt or albumin (in our assay), may increase the release of phospholipids simply by enhancing the solubilisation of the phospholipids from the outer canalicular membrane into the canaliculus.

Having set the conditions for our assay, we next tested the functional polarity of MDR3 by comparing transport of C₆-NBD-PC from the basolateral to the apical compartment with the transport from the apical to basolateral compartment. We found MDR3-mediated C₆-NBD-PC secretion exclusively in the apical compartment. This result is consistent with the previously shown immunolocalization of MDR3 on the apical membrane of BNMG cells. In conclusion, BNMG cells display vectorial transport (from basolateral to apical) of bile salt and phosphatidylcholine and hence recapitulate *in vitro* biliary phospholipid and bile salt secretion *in vivo* (Fig. 30).

5.1.6 Endogenous transporters with the same substrate specificity

Expression of endogenous transporters having the same substrate specificity as the tested transporters can provide a potential bias for *in vitro* studies. In the literature it has been reported that transfection of cells lines with transporters may alter endogenous MDR1 expression (Kuteykin-Teplyakov et al., 2010; Raggars et al., 2002). As previous experiments have shown that C₆-NBD-PC is also a substrate of MDR1 (Smith et al., 2000) and LLC-PK₁ cells have been reported to endogenously express little

amounts of pMDR1 (Goh et al., 2002), we analyzed endogenous pMDR1 expression by Western Blotting. Our results suggest that endogenous pMDR1 expression was minimal in WT LLC-PK₁ cells (Fig. 18), as compared with endogenous MDR1 expression in Caco-2 cells and that expression levels were similar in WT and BNMG cells, demonstrating that the difference of C₆-NBD-PC transport between BNMG cells and WT cells should reflect MDR3-mediated transport. The small amount of C₆-NBD-PC observed in the apical medium of WT cells could have been partially MDR1-mediated (by the very low amounts of pMDR1) and can partially be explained by the chemical properties of C₆-NBD-PC, which is more water soluble than PC. This partitioning into the aqueous phase is also illustrated by the basolateral release of C₆-NBD-PC in Fig. 17 and by the published observation that C₆-NBD-PC exchanges via the aqueous phase between artificial phospholipid vesicles (Elvington and Nichols, 2007).

5.1.7 Validation of the BNMG cell line for studying drug interactions with MDR3

Finally, the model for studying drug interaction with MDR3 was functionally validated by treating cells with two known reversal agents/inhibitors of MDR1 and MDR3 (Smith et al., 2000). Verapamil and PSC833 modulate the efflux function of MDR1 by competing for the substrate binding sites in the TMDs (Wu et al., 2008) and they have been shown to be extruded by MDR1. However, they do not seem to be extruded MDR3 (Morita et al., 2013; Wu et al., 2008). As expected, transport of C₆-NBD-PC was sensitive to verapamil and PSC833 suggesting that MDR3 was indeed inhibited by these drugs. However, a better demonstration that MDR3 inhibition was the reason for the decreased C₆-NBD-PC secretion, could have been provided by parallel treatment with verapamil and PSC833 of a control cell line, not expressing MDR3 (e.g. a BNG cell line expressing only BSEP, NTCP and ABCG5/G8).

The inhibition of MDR3 activity by PSC833 and verapamil was lower in our model system as compared to previous publications (van Helvoort et al., 1996; Yoshikado et al., 2011). It is likely that this difference is mainly due to the different experimental setup used.

Reasons that could underlie a lower inhibition in our model as compared to other models could include the use of the surrogate substrate C₆-NBD-PC having a lower affinity than the endogenous substrate PC, the presence of endogenous PC that may compete with C₆-NBD-PC and the way how the drug treatment was performed. C₆-NBD-PC possesses a different chemical structure and thus a different affinity to MDR3 than PC. This may also lead to different inhibition potency. In addition, in other assays drug treatment was performed by pre-incubating the cells before the start of the experiment and not simultaneous as we did. On the other hand, the higher inhibition found in other models could be due to perturbations of the biosynthetic pathway (when PC precursors were used as substrates) and to a high endogenous expression of MDRs such as MDR1 with the same substrate specificity. In the first case, perturbations of the biosynthetic pathways by the tested drug could result

in a lower amount of PC secreted even though MDR3 activity would be unchanged. In contrast, C₆-NBD-PC is independent from the cell biosynthetic pathway and therefore alterations of C₆-NBD-PC secretion should directly reflect alteration of MDR3 activity. Finally, different expression levels of endogenous transporters, or alteration of expression in transfected cell lines in other models may also lead to bias in the results as explained in the previous section.

5.2 Application of the established *in vitro* model for testing drugs interactions with hepatobiliary transporters

5.2.1 Effect of antifungal azoles on MDR3 and BSEP activities and comparison of their structures

Analysis of the FDA Adverse Event Reporting System database revealed that antimycotics are involved in approximately 3% of all DILI cases (Raschi et al., 2014). Moreover, antifungal azoles have been associated with an elevation of serum liver enzymes and liver injury leading in some cases to liver failure (Adriaenssens et al., 2001; Chang et al., 2007; Gearhart, 1994; Hann et al., 1993). Therefore, we tested the interaction of five clinically used antifungal azoles with MDR3: fluconazole, itraconazole, ketoconazole, posaconazole, voriconazole. Posaconazole, itraconazole and ketoconazole (with decreasing order) significantly inhibited MDR3-mediated phosphatidylcholine secretion and BSEP-mediated taurocholate secretion, as indicated by the reduction of C₆-NBD-PC secretion and the increased intracellular accumulation of taurocholate, in our model cell line. Posaconazole strongly inhibited MDR3 (83%, IC₅₀ ~ 4.2 µM) and moderately inhibited BSEP (38%). This is, to our knowledge, the first report on the inhibition of canalicular transporters, particularly MDR3, by posaconazole. In clinics posaconazole-related elevation of liver enzymes or liver injury were rarely reported and most available data are in the context of clinical trials (Courtney et al., 2005; Girmenia, 2009; Moton et al., 2009). The apparent discrepancy between our findings and the current literature may have several reasons: 1) posaconazole was approved in 2006 and is the newest azole released into the market about 20 years after ketoconazole and itraconazole. 2) Posaconazole has a rather narrow indication, e.g. for the treatment of invasive fungal infections (e.g. aspergillosis, candidiasis) in patients refractory to other antifungals (second line therapy) and for the prophylaxis of antifungal infections in patients receiving chemotherapy and immunosuppressed patients after hematopoietic-stem-cell-transplantation (Frampton and Scott, 2008). Hence, the patient population exposed to posaconazole is limited and often is on polypharmacy. In such situations it is difficult to associate a particular drug with a specific molecular pathway leading to the adverse reaction. Similarly to posaconazole, also itraconazole significantly inhibited MDR3 (42%) and moderately inhibited BSEP (26%). These results confirm previous studies (He et al., 2015; Yoshikado et al., 2011) and are clinically supported by the numerous reports of hepatic liver injury present in the literature (Chang et al., 2007; Hann et al., 1993; Hay, 1993) and in particular reports on a cholestatic pattern of liver injury (Hann et al., 1993; Lavrijsen et al., 1992; Talwalkar et al., 1999; Yoshikado et al., 2011). Interestingly, ketoconazole only weakly (20%) inhibited MDR3 but was in the present study the most potent BSEP inhibitor (67% inhibition). This BSEP inhibition is in line with previous reports of BSEP inhibition by ketoconazole (IC₅₀ ~ 3 µM (Dawson et al., 2012)). Ketoconazole has been

withdrawn from the European and Australian markets and underwent strict product relabeling in Canada and US due to hepatotoxicity (Gupta et al., 2015). Although these adverse effects were mainly attributed to the inhibition of CYP3A isoforms (Phase I enzymes) by ketoconazole, emerging evidence (Dawson et al., 2012; Morgan et al., 2010) and our results suggest that inhibition of MDR3 in combination with BSEP in susceptible patients may aggravate ketoconazole-induced hepatotoxicity similarly to troglitazone (Stieger, 2010). Fluconazole and voriconazole did neither affect MDR3-mediated PC secretion nor BSEP in our model system. These results agree well with several comparative *in vitro* and epidemiological studies suggesting that fluconazole is the safest azole (Chang et al., 2007; Cronin and Chandrasekar, 2010; Somchit et al., 2002). In contrast, voriconazole has been often associated to hepatotoxicity. However, its toxicity seems to correlate with CYP2C19 polymorphisms (Trubiano et al., 2015) and not to inhibition of hepatobiliary transporters. Interestingly, the azoles showing an effect on the canalicular transporters have a structural resemblance (Fig. 31). The MDR3 inhibitors posaconazole and itraconazole contain an extended piperazine-phenyl-triazole moiety while ketoconazole contains a piperazine-phenyl moiety. In contrast, voriconazole and fluconazole did not inhibit MDR3 and are structurally different (voriconazole was developed by chemical manipulation of fluconazole). Therefore, it is tempting to speculate that the piperazine-phenyl-triazole moiety is responsible for the interaction with MDR3. Although additional experiments e.g. using vesicle-based studies (ATPase assay) would be required to prove direct binding of these drugs to BSEP and MDR3, the parallel inhibition of these proteins by posaconazole, itraconazole and ketoconazole could suggest that these azoles may bind conserved domains of MDR3 and BSEP. A comparison of the primary amino acid sequence of BSEP and MDR3 reveals nearly 50% sequence identity in the entire proteins but 65% sequence identity in the NBDs. Hence, one could speculate that these drugs may inhibit the ATPase activity of BSEP and MDR3. Structural information on MDR3 and BSEP will help to elucidate the nature (e.g. binding site prediction) of this interaction at the molecular level. Furthermore, in the future (with the availability of the protein structures), using a computational structure-based drug design approach, posaconazole and itraconazole or the piperazine-phenyl-triazole moiety could be used as hit for the identification of MDR3 modulators.

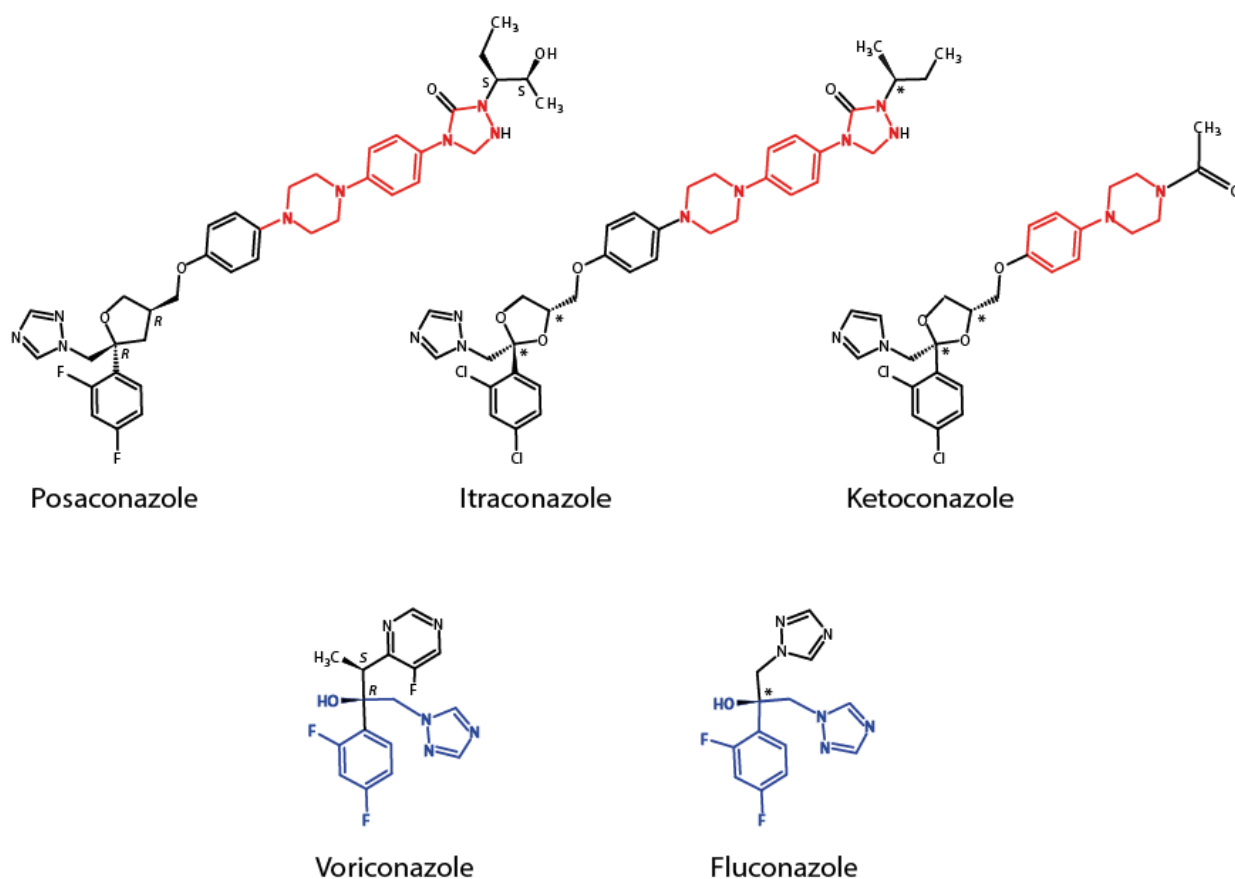


Fig. 31 Chemical structures of the azoles tested in our *in vitro* model. Antifungal azoles act by inhibiting the conversion of lanosterol to ergosterol, the main sterol in fungal cell membrane. Their chemical structure is characterized by five-membered rings containing two (imidazoles) or three (triazoles) nitrogen atoms. Posaconazole, itraconazole and ketoconazole share common structures. The piperazine-phenyl-triazole moiety of posaconazole and itraconazole as well as the piperazine-phenyl moiety of ketoconazole are depicted in red. Similarly, fluconazole and voriconazole share also several functional groups as depicted in blue.

5.2.2 Effect of antifungal azoles on MDR3 protein levels

Very interestingly, itraconazole and posaconazole led to a marked time-dependent increase in MDR3 protein levels in our model system. No significant change in BSEP and NTCP (Fig. 23A and Fig. 23B) protein levels was detected under the same condition. As we used cDNAs to generate our model cell line, an upregulation of the transcription should not be the reason for this. This is also further supported by the observation that the PPAR α agonists fenofibrate and bezafibrate, known to stimulate MDR3 promoter activity (Ghonem et al., 2014), did not lead to a change of MDR3 protein levels in our model cell line (Fig. 23B). We did not see an increase of mRNA for MDR3 (Fig. 25), ruling out a stabilization of the mRNA by these two drugs. In addition to the marked increase of MDR3 protein levels, in the Western blots it is observable a slight lower MDR3 band in cells treated with posaconazole and itraconazole, suggesting that the increase may involve the de-glycosylated form MDR3. N-glycosylation has been demonstrated to play a critical role in many biological processes,

e.g. in protein folding and in maintenance of stability, in the regulation of intracellular targeting of proteins and also in apical targeting of membrane proteins (Draheim et al., 2010). In a study it has been shown that treating hepatocytes with tunicamycin, an inhibitor of glycosylation, decreased N-glycosylation and simultaneously impaired the trafficking of MRP2 to the canalicular membrane (Zhang et al., 2005). This would lead to the speculation that similarly to tunicamycin, itraconazole and posaconazole by inhibiting N-glycosylation, and impairing apical targeting of MDR3, may inhibit MDR3-mediated C₆-NBD-PC secretion as shown by our results. Another possible explanation for these observations could be direct binding of posaconazole and itraconazole to MDR3 followed by protein inhibition and stabilization. MDR3 stabilization may cause a reduction of its degradation with a consequent intracellular accumulation of de-glycosylated MDR3. Finally, also an increased translation rate may lead to the increased intracellular accumulation of de-glycosylated protein and a general rise of MDR3 protein levels. Elucidation of the exact molecular mechanisms of these observations would require additional studies.

5.2.3 Effect of additional hepatotoxic drugs on MDR3 activity

Several additional drugs associated with cholestatic DILI were tested in our model system and showed no effect (with exception of cyclosporin A). Of these drugs, co-amoxicillin (amoxicillin–clavulanate), chlorpromazine and nefadozone have been implicated in liver failures and in several cases of DILI in at least 3 countries registries (Spanish, Swedish and American) (Suzuki et al., 2010). Amoxicillin–clavulanate is among the most commonly prescribed antibiotics worldwide and it is the drug which has been most implicated with DILI (1 case out of 2300 users have been estimated) (Bjornsson, 2015). The lack of effect of this drug on MDR3-mediated phospholipid secretion is consistent with the immunoallergic pathogenic mechanism proposed for this drug. Co-amoxicillin-induced liver injury manifests generally as cholestatic hepatitis, together with fever, rash and eosinophilia. Similarly to co-amoxicillin, chlorpromazine induced hepatotoxicity has been associated with an immunoallergic reaction (LiverTox.nih.gov[2016]). On the mechanism leading to nefadozone-induced hepatotoxicity, less is known, but as nefadozone is extensively metabolized by P450 enzymes generation of toxic metabolites may be involved. This illustrates the complexity and the multifactorial nature of DILI. In addition, the inhibition by drugs detected in our *in vitro* model system seems to be specific and suggests that our *in vitro* setup does not render MDR3 susceptible to pathophysiological irrelevant inhibitions by drugs as a consequence of a lipid and protein environment different from the canalicular membrane.

5.2.4 Effect of cholagogues and fibrates on MDR3 activity

Cholagogues are traditionally used to enhance bile secretion and fibrates have been reported to enhance MDR3-mediated transport, therefore we tested in our *in vitro* model if these compounds may enhance MDR3-mediated C₆-NBD-PC secretion. Molecular mechanisms underlying such an enhancement could possibly involve 1) transcriptional upregulation of transporter expression, 2) protein stabilization on the membrane 3) cellular re-distribution of the transporter/apical sorting of the transporter 4) reduction of protein turnover/protein degradation. Considering that our *in vitro* model constitutively expresses MDR3, it is clear that it is not possible to examine transcriptional regulation of MDR3 in our BNMG cell line. However, the other mechanisms could be examined. In our model system neither cholagogues nor fibrates enhanced MDR3-mediated C₆-NBD-PC secretion. The little reproducibility of the fibrates results could be due to a solubility problem as after adding the stock solution (in DMSO) to the medium, formation of precipitates was observed under the microscope. However, it is not clear from the literature how this technical issue was managed in previous *in vitro* studies. It may be possible that the precipitation resulted from an interaction with a specific component of the medium used for BNMG cells.

5.2.5 Importance of transport proteins in drug development

In the last decades, the role of transporters as determinants of drug pharmacokinetic and drug-drug interactions, in addition to drug metabolizing enzymes, has been increasingly recognized by the regulatory agencies and the pharmaceutical industry (Brouwer et al., 2013; International Transporter et al., 2010; Konig et al., 2013). As illustrated in this thesis, inhibition of drug transporters might not only play a significant role on drug pharmacokinetic and drug efficacy of multimorbid and polymedicated patients (such as elderly patients) but may also affect the fate of endogenous molecules such as lipids, bilirubin and bile salts, posing significant potential safety liabilities (Funk, 2008). Accordingly, in both new drug-drug interactions guidelines issued by FDA and EMA there is an increased emphasis on transporter based drug-drug interaction evaluations, and in both guidelines the list of transporters considered as clinical relevant and recommended for *in vitro* inhibition studies has grown (Prueksaritanont et al., 2013). In this context, many *in vitro* tools have been and are being developed to study and predict drug transporters interactions or to clarify the mechanism of unexpected interactions observed *in vivo*. However, to date only few studies have addressed the question of the role of lipid transporters in drug interactions. In this work, we identified among the clinically used drugs that were tested a potent inhibitor of phospholipid secretion. Future work is required to understand the clinical significance or impact of this interaction in humans.

5.2.6 Perspective

In this thesis an *in vitro* assay to study selected transporters implicated in DILI and their inhibition by different drugs has been tested. Studying the interaction of drugs with transporters in our *in vitro* model, we found that parallel inhibition of BSEP and MDR3 may underlie ketoconazole, itraconazole and posaconazole induced liver injury. In addition, based on the literature, generation of toxic metabolites is also implicated in itraconazole- and ketoconazole- induced liver injury (Somchit et al., 2004). Similarly to this case, troglitazone-induced liver injury has been attributed to an inhibition of BSEP, as well as to mitochondrial dysfunction by induction of oxidative stress. These examples show that the pathogenesis of DILI is complex and that DILI may results by the interplay of several simultaneous pathological mechanisms that can be sequentially not linked between each other (Roth and Ganey, 2010). This complexity is also supported by the difficulty of diagnosing DILI. This diagnosis is made by exclusion (e.g. other potential causes leading to liver disease are excluded). Therefore, better diagnostic markers for the different types of DILI would be important to classify patients and to guide future clinical research. In addition, a computational biology-based approach aiming to integrate qualitative and quantitative data generated from *in vitro/vivo* models and/or human clinical data (when available) into a comprehensive mechanistic model, that explains the contribution of different pathological pathways to DILI, could be helpful to improve our understanding and prediction of DILI (de Lima Toccafondo Vieira and Tagliati, 2014).

Besides progress in the direction of better clinical and mechanistic understanding of the disease, a better molecular understanding of the underlying drug-protein interactions, leading to the activation of pathological pathways, would be crucial to develop safer drugs. A prerequisite for this would be a better structural understanding of the involved proteins e.g. ABC transporters. Currently no structure is available for MDR3 and BSEP. Recently a structure for ABCG5/G8 has been published (Lee et al., 2016). With structural information available, a computational structure-based approach could then be used to design and finally develop safer drugs and/or modulators that could be used for future research.

6 References

- Adriaenssens B, Roskams T, Steger P and Van Steenberghe W (2001) Hepatotoxicity related to itraconazole: report of three cases. *Acta clinica Belgica* **56**(6): 364-369.
- Adzet T, Camarasa J and Laguna JC (1987) Hepatoprotective Activity of Polyphenolic Compounds from *Cynara-Scolymus* against Ccl4 Toxicity in Isolated Rat Hepatocytes. *Journal of Natural Products* **50**(4): 612-617.
- Aithal GP, Watkins PB, Andrade RJ, Larrey D, Molokhia M, Takikawa H, Hunt CM, Wilke RA, Avigan M, Kaplowitz N, Bjornsson E and Daly AK (2011) Case definition and phenotype standardization in drug-induced liver injury. *Clinical pharmacology and therapeutics* **89**(6): 806-815.
- Alvaro D, Cantafora A, Attili AF, Ginanni Corradini S, De Luca C, Minervini G, Di Biase A and Angelico M (1986) Relationships between bile salts hydrophilicity and phospholipid composition in bile of various animal species. *Comparative biochemistry and physiology B, Comparative biochemistry* **83**(3): 551-554.
- Amacher DE and Chalasani N (2014) Drug-induced hepatic steatosis. *Seminars in liver disease* **34**(2): 205-214.
- avantilipids.com (2016) Avanti Polar Lipids, Inc., AL, USA
- Barone GW, Gurley BJ, Ketel BL, Lightfoot ML and Abul-Ezz SR (2000) Drug interaction between St. John's wort and cyclosporine. *The Annals of pharmacotherapy* **34**(9): 1013-1016.
- Bhamidimarri KR and Schiff E (2013) Drug-induced cholestasis. *Clinics in liver disease* **17**(4): 519-531, vii.
- Bjornsson ES (2015) Drug-induced liver injury: an overview over the most critical compounds. *Archives of toxicology* **89**(3): 327-334.
- Bjornsson ES, Bergmann OM, Bjornsson HK, Kvaran RB and Olafsson S (2013) Incidence, presentation, and outcomes in patients with drug-induced liver injury in the general population of Iceland. *Gastroenterology* **144**(7): 1419-1425, 1425 e1411-1413; quiz e1419-1420.
- Bligh EG and Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology* **37**(8): 911-917.
- Boyer JL (2013) Bile formation and secretion. *Comprehensive Physiology* **3**(3): 1035-1078.
- Brouwer KL, Keppler D, Hoffmaster KA, Bow DA, Cheng Y, Lai Y, Palm JE, Stieger B, Evers R and International Transporter C (2013) In vitro methods to support transporter evaluation in drug discovery and development. *Clinical pharmacology and therapeutics* **94**(1): 95-112.

- Buschman E, Arceci RJ, Croop JM, Che M, Arias IM, Housman DE and Gros P (1992) *mdr2* encodes P-glycoprotein expressed in the bile canalicular membrane as determined by isoform-specific antibodies. *The Journal of biological chemistry* **267**(25): 18093-18099.
- Cao X, Surma MA and Simons K (2012) Polarized sorting and trafficking in epithelial cells. *Cell Res* **22**(5): 793-805.
- Chang CH, Young-Xu Y, Kurth T, Orav JE and Chan AK (2007) The safety of oral antifungal treatments for superficial dermatophytosis and onychomycosis: a meta-analysis. *The American journal of medicine* **120**(9): 791-798.
- Cheng F, Theodorescu D, Schulman IG and Lee JK (2011) In vitro transcriptomic prediction of hepatotoxicity for early drug discovery. *Journal of theoretical biology* **290**: 27-36.
- Chughlay M, Blockman M and Cohen K (2015) A clinical approach to drug-induced liver injury. *Current Allergy & Clinical Immunology* **28**(4).
- Corsini A and Bortolini M (2013) Drug-induced liver injury: the role of drug metabolism and transport. *Journal of clinical pharmacology* **53**(5): 463-474.
- Courtney R, Sansone A, Smith W, Marbury T, Statkevich P, Martinho M, Laughlin M and Swan S (2005) Posaconazole pharmacokinetics, safety, and tolerability in subjects with varying degrees of chronic renal disease. *Journal of clinical pharmacology* **45**(2): 185-192.
- Crivellato E, Candussio L, Rosati AM, Decorti G, Klugmann FB and Mallardi F (1999) Kinetics of doxorubicin handling in the LLC-PK1 kidney epithelial cell line is mediated by both vesicle formation and P-glycoprotein drug transport. *The Histochemical journal* **31**(10): 635-643.
- Cronin S and Chandrasekar PH (2010) Safety of triazole antifungal drugs in patients with cancer. *The Journal of antimicrobial chemotherapy* **65**(3): 410-416.
- Cuthbertson L, Kos V and Whitfield C (2010) ABC transporters involved in export of cell surface glycoconjugates. *Microbiol Mol Biol Rev* **74**(3): 341-362.
- Dawson S, Stahl S, Paul N, Barber J and Kenna JG (2012) In vitro inhibition of the bile salt export pump correlates with risk of cholestatic drug-induced liver injury in humans. *Drug metabolism and disposition: the biological fate of chemicals* **40**(1): 130-138.
- de Lima Toccafondo Vieira M and Tagliati CA (2014) Hepatobiliary transporters in drug-induced cholestasis: a perspective on the current identifying tools. *Expert opinion on drug metabolism & toxicology* **10**(4): 581-597.
- Dean M (2002) *The Human ATP-Binding Cassette (ABC) Transporter Superfamily*. National Center for Biotechnology Information, US.
- Derby LE, Gutthann SP, Jick H and Dean AD (1993) Liver disorders in patients receiving chlorpromazine or isoniazid. *Pharmacotherapy* **13**(4): 353-358.

- Devarbhavi H, Dierkhising R, Kremers WK, Sandeep MS, Karanth D and Adarsh CK (2010) Single-Center Experience With Drug-Induced Liver Injury From India: Causes, Outcome, Prognosis, and Predictors of Mortality. *American Journal of Gastroenterology* **105**(11): 2396-2404.
- Dobson PD and Kell DB (2008) Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nature reviews Drug discovery* **7**(3): 205-220.
- Doring B and Petzinger E (2014) Phase 0 and phase III transport in various organs: combined concept of phases in xenobiotic transport and metabolism. *Drug metabolism reviews* **46**(3): 261-282.
- Dowling RH, Hussaini SH, Murphy GM, Besser GM and Wass JA (1992) Gallstones during octreotide therapy. *Metabolism: clinical and experimental* **41**(9 Suppl 2): 22-33.
- Draheim V, Reichel A, Weitschies W and Moenning U (2010) N-glycosylation of ABC transporters is associated with functional activity in sandwich-cultured rat hepatocytes. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences* **41**(2): 201-209.
- Ellinger P, Kluth M, Stindt J, Smits SH and Schmitt L (2013) Detergent screening and purification of the human liver ABC transporters BSEP (ABCB11) and MDR3 (ABCB4) expressed in the yeast *Pichia pastoris*. *PloS one* **8**(4): e60620.
- Elvington SM and Nichols JW (2007) Spontaneous, intervesicular transfer rates of fluorescent, acyl chain-labeled phosphatidylcholine analogs. *Biochimica et biophysica acta* **1768**(3): 502-508.
- Farrell GC (1994) *Drug-induced liver disease*. Longman Singapore Publishers, Singapore.
- Fontana RJ (2014) Pathogenesis of idiosyncratic drug-induced liver injury and clinical perspectives. *Gastroenterology* **146**(4): 914-928.
- Frampton JE and Scott LJ (2008) Posaconazole : a review of its use in the prophylaxis of invasive fungal infections. *Drugs* **68**(7): 993-1016.
- Fujikura K, Ingelman-Sundberg M and Lauschke VM (2015) Genetic variation in the human cytochrome P450 supergene family. *Pharmacogenetics and genomics* **25**(12): 584-594.
- Funk C (2008) The role of hepatic transporters in drug elimination. *Expert opinion on drug metabolism & toxicology* **4**(4): 363-379.
- Gearhart MO (1994) Worsening of liver function with fluconazole and review of azole antifungal hepatotoxicity. *The Annals of pharmacotherapy* **28**(10): 1177-1181.
- Gerloff T, Meier PJ and Stieger B (1998) Taurocholate induces preferential release of phosphatidylcholine from rat liver canalicular vesicles. *Liver* **18**(5): 306-312.
- Ghonem NS, Ananthanarayanan M, Soroka CJ and Boyer JL (2014) Peroxisome proliferator-activated receptor alpha activates human multidrug resistance transporter 3/ATP-binding cassette protein subfamily B4 transcription and increases rat biliary phosphatidylcholine secretion. *Hepatology* **59**(3): 1030-1042.

- Girmenia C (2009) New generation azole antifungals in clinical investigation. *Expert opinion on investigational drugs* **18**(9): 1279-1295.
- Goh LB, Spears KJ, Yao D, Ayrton A, Morgan P, Roland Wolf C and Friedberg T (2002) Endogenous drug transporters in in vitro and in vivo models for the prediction of drug disposition in man. *Biochemical pharmacology* **64**(11): 1569-1578.
- Gonzalez RJ and Tarloff JB (2004) Expression and activities of several drug-metabolizing enzymes in LLC-PK1 cells. *Toxicology in vitro : an international journal published in association with BIBRA* **18**(6): 887-894.
- Greer ML, Barber J, Eakins J and Kenna JG (2010) Cell based approaches for evaluation of drug-induced liver injury. *Toxicology* **268**(3): 125-131.
- Grenier E, Garofalo C, Delvin E and Levy E (2012) Modulatory role of PYY in transport and metabolism of cholesterol in intestinal epithelial cells. *PloS one* **7**(7): e40992.
- Gupta AK, Daigle D and Foley KA (2015) Drug safety assessment of oral formulations of ketoconazole. *Expert opinion on drug safety* **14**(2): 325-334.
- Hagenbuch B and Stieger B (2013) The SLCO (former SLC21) superfamily of transporters. *Molecular aspects of medicine* **34**(2-3): 396-412.
- Hann SK, Kim JB, Im S, Han KH and Park YK (1993) Itraconazole-Induced Acute Hepatitis. *Brit J Dermatol* **129**(4): 500-501.
- Hay RJ (1993) Risk/benefit ratio of modern antifungal therapy: focus on hepatic reactions. *Journal of the American Academy of Dermatology* **29**(1): S50-54.
- He K, Cai L, Shi Q, Liu H and Woolf TF (2015) Inhibition of MDR3 Activity in Human Hepatocytes by Drugs Associated with Liver Injury. *Chemical research in toxicology* **28**(10): 1987-1990.
- Hidalgo IJ, Raub TJ and Borchardt RT (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **96**(3): 736-749.
- Higgins CF and Linton KJ (2004) The ATP switch model for ABC transporters. *Nature structural & molecular biology* **11**(10): 918-926.
- Ho RH, Tirona RG, Leake BF, Glaeser H, Lee W, Lemke CJ, Wang Y and Kim RB (2006) Drug and bile acid transporters in rosuvastatin hepatic uptake: function, expression, and pharmacogenetics. *Gastroenterology* **130**(6): 1793-1806.
- Holland IB, Cole SPC, Kuchler K and Higgins CF (2003) *ABC Proteins: From Bacteria to Man*. Academic Press, London.
- Hollenstein K, Dawson RJ and Locher KP (2007) Structure and mechanism of ABC transporter proteins. *Curr Opin Struct Biol* **17**(4): 412-418.
- Hosoya KI, Kim KJ and Lee VH (1996) Age-dependent expression of P-glycoprotein gp170 in Caco-2 cell monolayers. *Pharmaceutical research* **13**(6): 885-890.

- Huang L, Hoffman T and Vore M (1998) Adenosine triphosphate-dependent transport of estradiol-17 β (β -D-glucuronide) in membrane vesicles by MDR1 expressed in insect cells. *Hepatology* **28**(5): 1371-1377.
- Hussaini SH and Farrington EA (2007) Idiosyncratic drug-induced liver injury: an overview. *Expert opinion on drug safety* **6**(6): 673-684.
- Hussaini SH and Farrington EA (2014) Idiosyncratic drug-induced liver injury: an update on the 2007 overview. *Expert opinion on drug safety* **13**(1): 67-81.
- Ingelman-Sundberg M (2004) Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms. *Naunyn-Schmiedeberg's archives of pharmacology* **369**(1): 89-104.
- International Transporter C, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, Ishikawa T, Keppler D, Kim RB, Lee CA, Niemi M, Polli JW, Sugiyama Y, Swaan PW, Ware JA, Wright SH, Yee SW, Zamek-Gliszczynski MJ and Zhang L (2010) Membrane transporters in drug development. *Nature reviews Drug discovery* **9**(3): 215-236.
- Jacquemin E (2012) Progressive familial intrahepatic cholestasis. *Clinics and research in hepatology and gastroenterology* **36 Suppl 1**: S26-35.
- Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D and Lemasters JJ (2002) Mechanisms of hepatotoxicity. *Toxicological sciences : an official journal of the Society of Toxicology* **65**(2): 166-176.
- Johansson I and Ingelman-Sundberg M (2011) Genetic polymorphism and toxicology--with emphasis on cytochrome p450. *Toxicological sciences : an official journal of the Society of Toxicology* **120**(1): 1-13.
- Ju C and Reilly T (2012) Role of immune reactions in drug-induced liver injury (DILI). *Drug metabolism reviews* **44**(1): 107-115.
- Jungermann K (1987) Metabolic zonation of liver parenchyma: significance for the regulation of glycogen metabolism, gluconeogenesis, and glycolysis. *Diabetes Metab Rev* **3**(1): 269-293.
- Kaplowitz N (2004) Drug-induced liver injury. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **38 Suppl 2**: S44-48.
- Kaplowitz N and DeLeve LD (2003) *Drug-induced liver disease*. New York: Marcel Dekker.
- Kaplowitz N and DeLeve LD (2013) *Drug-induced liver disease*. Third edition ed. Academic Press.
- Kirchhoff R, Beckers C, Kirchhoff GM, Trinczek-Gartner H, Petrowicz O and Reimann HJ (1994) Increase in cholereresis by means of artichoke extract. *Phytomedicine* **1**(2): 107-115.
- Klaassen C (2008) *Casarett & Doull's Toxicology: the basic science of poison*. Seventh ed.
- Klaassen CD and Watkins III JB (2010) *Casarett & Doull's Essentials of Toxicology*. Second ed. McGraw-Hill.

- Kock K, Ferslew BC, Netterberg I, Yang K, Urban TJ, Swaan PW, Stewart PW and Brouwer KL (2014) Risk factors for development of cholestatic drug-induced liver injury: inhibition of hepatic basolateral bile acid transporters multidrug resistance-associated proteins 3 and 4. *Drug metabolism and disposition: the biological fate of chemicals* **42**(4): 665-674.
- Konig J, Muller F and Fromm MF (2013) Transporters and drug-drug interactions: important determinants of drug disposition and effects. *Pharmacological reviews* **65**(3): 944-966.
- Kraft K (1997) Artichoke leaf extract - Recent findings reflecting effects on lipid metabolism, liver and gastrointestinal tracts. *Phytomedicine* **4**(4): 369-378.
- Kuteykin-Teplyakov K, Luna-Tortos C, Ambroziak K and Loscher W (2010) Differences in the expression of endogenous efflux transporters in MDR1-transfected versus wildtype cell lines affect P-glycoprotein mediated drug transport. *British journal of pharmacology* **160**(6): 1453-1463.
- Labbe G, Pessayre D and Fromenty B (2008) Drug-induced liver injury through mitochondrial dysfunction: mechanisms and detection during preclinical safety studies. *Fundamental & clinical pharmacology* **22**(4): 335-353.
- Lavrijssen AP, Balmus KJ, Nugteren-Huying WM, Roldaan AC, van't Wout JW and Stricker BH (1992) Hepatic injury associated with itraconazole. *Lancet* **340**(8813): 251-252.
- Lee JY, Kinch LN, Borek DM, Wang J, Wang J, Urbatsch IL, Xie XS, Grishin NV, Cohen JC, Otwinowski Z, Hobbs HH and Rosenbaum DM (2016) Crystal structure of the human sterol transporter ABCG5/ABCG8. *Nature* **533**(7604): 561-564.
- Leise MD, Poterucha JJ and Talwalkar JA (2014) Drug-induced liver injury. *Mayo Clinic proceedings* **89**(1): 95-106.
- Lewis JH (2000) Drug-induced liver disease. *Med Clin North Am* **84**(5): 1275-1311, x.
- Liu J, Kesiry R, Periyasamy SM, Malhotra D, Xie Z and Shapiro JI (2004) Ouabain induces endocytosis of plasmalemmal Na/K-ATPase in LLC-PK1 cells by a clathrin-dependent mechanism. *Kidney international* **66**(1): 227-241.
- LiverTox.nih.gov[2016] LiverTox: Clinical and Research Information on Drug-Induced Liver Injury, NIH, U.S National Library of Medicine NLM and NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases
- Locher KP (2009) Review. Structure and mechanism of ATP-binding cassette transporters. *Philos Trans R Soc Lond B Biol Sci* **364**(1514): 239-245.
- Locher KP (2016) Mechanistic diversity in ATP-binding cassette (ABC) transporters. *Nature structural & molecular biology* **23**(6): 487-493.
- Mahdi ZM, Synal-Hermanns U, Yoker A, Locher KP and Stieger B (2016) Role of Multidrug Resistance Protein 3 (MDR3) in Antifungal-Induced Cholestasis. *Molecular pharmacology*.

- Masubuchi Y, Kano S and Horie T (2006) Mitochondrial permeability transition as a potential determinant of hepatotoxicity of antidiabetic thiazolidinediones. *Toxicology* **222**(3): 233-239.
- Matuschowski P, Gumbinger HG and Winterhoff H (1997) Influence of *Cynara scolymus* L on bile flow and bile acid production in the isolated perfused rat liver. *N-S Arch Pharmacol* **355**(4): 398-398.
- McCuskey R (2012) Pathophysiology of the Liver, Chapter: Anatomy of the Liver, in *Zakim and Boyer's Hepatology: A Textbook of Liver Disease* (Boyer TD, Manns MP and Sanyal AJ eds), Elsevier.
- Morgan RE, Trauner M, van Staden CJ, Lee PH, Ramachandran B, Eschenberg M, Afshari CA, Qualls CW, Jr., Lightfoot-Dunn R and Hamadeh HK (2010) Interference with bile salt export pump function is a susceptibility factor for human liver injury in drug development. *Toxicological sciences: an official journal of the Society of Toxicology* **118**(2): 485-500.
- Morgan RE, van Staden CJ, Chen Y, Kalyanaraman N, Kalanzi J, Dunn RT, 2nd, Afshari CA and Hamadeh HK (2013) A multifactorial approach to hepatobiliary transporter assessment enables improved therapeutic compound development. *Toxicological sciences : an official journal of the Society of Toxicology* **136**(1): 216-241.
- Morita SY, Kobayashi A, Takanezawa Y, Kioka N, Handa T, Arai H, Matsuo M and Ueda K (2007) Bile salt-dependent efflux of cellular phospholipids mediated by ATP binding cassette protein B4. *Hepatology* **46**(1): 188-199.
- Morita SY, Tsuda T, Horikami M, Teraoka R, Kitagawa S and Terada T (2013) Bile salt-stimulated phospholipid efflux mediated by ABCB4 localized in nonraft membranes. *Journal of lipid research* **54**(5): 1221-1230.
- Moton A, Krishna G and Wang Z (2009) Tolerability and safety profile of posaconazole: evaluation of 18 controlled studies in healthy volunteers. *Journal of clinical pharmacy and therapeutics* **34**(3): 301-311.
- Nicolaou M, Andress EJ, Zolnerciks JK, Dixon PH, Williamson C and Linton KJ (2012) Canalicular ABC transporters and liver disease. *The Journal of pathology* **226**(2): 300-315.
- Niemi M (2007) Role of OATP transporters in the disposition of drugs. *Pharmacogenomics* **8**(7): 787-802.
- O'Brien P, Carrasco-Pozo C and Speisky H (2006) Boldine and its antioxidant or health-promoting properties. *Chemico-biological interactions* **159**(1): 1-17.
- Obaidat A, Roth M and Hagenbuch B (2012) The expression and function of organic anion transporting polypeptides in normal tissues and in cancer. *Annu Rev Pharmacol Toxicol* **52**: 135-151.
- Ogu CC and Maxa JL (2000) Drug interactions due to cytochrome P450. *Proceedings* **13**(4): 421-423.

- Ohno H, Tomemori T, Nakatsu F, Okazaki Y, Aguilar RC, Foelsch H, Mellman I, Saito T, Shirasawa T and Bonifacino JS (1999) Mu1B, a novel adaptor medium chain expressed in polarized epithelial cells. *FEBS letters* **449**(2-3): 215-220.
- Park SH and Ishino R (2013) Liver injury associated with antidepressants. *Curr Drug Saf* **8**(3): 207-223.
- Pauli-Magnus C and Meier PJ (2006) Hepatobiliary transporters and drug-induced cholestasis. *Hepatology* **44**(4): 778-787.
- Pauli-Magnus C, Meier PJ and Stieger B (2010) Genetic determinants of drug-induced cholestasis and intrahepatic cholestasis of pregnancy. *Seminars in liver disease* **30**(2): 147-159.
- Perez C, Gerber S, Boilevin J, Bucher M, Darbre T, Aebi M, Reymond JL and Locher KP (2015) Structure and mechanism of an active lipid-linked oligosaccharide flippase. *Nature* **524**(7566): 433-438.
- Phillips GB (1960) The lipid composition of human bile. *Biochimica et biophysica acta* **41**: 361-363.
- Prueksaritanont T, Chu X, Gibson C, Cui D, Yee KL, Ballard J, Cabalu T and Hochman J (2013) Drug-drug interaction studies: regulatory guidance and an industry perspective. *The AAPS journal* **15**(3): 629-645.
- Raggers RJ, Vogels I and van Meer G (2002) Upregulation of the expression of endogenous Mdr1 P-glycoprotein enhances lipid translocation in MDCK cells transfected with human MRP2. *Histochemistry and cell biology* **117**(2): 181-185.
- Ramachandran R and Kakar S (2009) Histological patterns in drug-induced liver disease. *Journal of clinical pathology* **62**(6): 481-492.
- Raschi E, Poluzzi E, Koci A, Caraceni P and Ponti FD (2014) Assessing liver injury associated with antimycotics: Concise literature review and clues from data mining of the FAERS database. *World journal of hepatology* **6**(8): 601-612.
- Reuben A, Koch DG, Lee WM and Acute Liver Failure Study G (2010) Drug-induced acute liver failure: results of a U.S. multicenter, prospective study. *Hepatology* **52**(6): 2065-2076.
- Roth RA and Ganey P (2010) Volume 9: Hepatic Toxicology, in *Comprehensive Toxicology*, Elsevier.
- Rudkowska I and Jones PJ (2008) Polymorphisms in ABCG5/G8 transporters linked to hypercholesterolemia and gallstone disease. *Nutr Rev* **66**(6): 343-348.
- Ruetz S and Gros P (1994) Phosphatidylcholine translocase: a physiological role for the mdr2 gene. *Cell* **77**(7): 1071-1081.
- Ruetz S and Gros P (1995) Enhancement of Mdr2-mediated phosphatidylcholine translocation by the bile salt taurocholate. Implications for hepatic bile formation. *The Journal of biological chemistry* **270**(43): 25388-25395.

- Salem MB, Affes H, Ksouda K, Dhouibi R, Sahnoun Z, Hammami S and Zeghal KM (2015) Pharmacological Studies of Artichoke Leaf Extract and Their Health Benefits. *Plant foods for human nutrition*.
- Scheffer GL, Kool M, Heijn M, de Haas M, Pijnenborg AC, Wijnholds J, van Helvoort A, de Jong MC, Hooijberg JH, Mol CA, van der Linden M, de Vree JM, van der Valk P, Elferink RP, Borst P and Scheper RJ (2000) Specific detection of multidrug resistance proteins MRP1, MRP2, MRP3, MRP5, and MDR3 P-glycoprotein with a panel of monoclonal antibodies. *Cancer research* **60**(18): 5269-5277.
- Shoda J, Inada Y, Tsuji A, Kusama H, Ueda T, Ikegami T, Suzuki H, Sugiyama Y, Cohen DE and Tanaka N (2004) Bezafibrate stimulates canalicular localization of NBD-labeled PC in HepG2 cells by PPARalpha-mediated redistribution of ABCB4. *Journal of lipid research* **45**(10): 1813-1825.
- Shoda J, Okada K, Inada Y, Kusama H, Utsunomiya H, Oda K, Yokoi T, Yoshizato K and Suzuki H (2007) Bezafibrate induces multidrug-resistance P-Glycoprotein 3 expression in cultured human hepatocytes and humanized livers of chimeric mice. *Hepatology research : the official journal of the Japan Society of Hepatology* **37**(7): 548-556.
- Shugarts S and Benet LZ (2009) The role of transporters in the pharmacokinetics of orally administered drugs. *Pharmaceutical research* **26**(9): 2039-2054.
- Small DM (2003) Role of ABC transporters in secretion of cholesterol from liver into bile. *Proceedings of the National Academy of Sciences of the United States of America* **100**(1): 4-6.
- Smit JJ, Schinkel AH, Oude Elferink RP, Groen AK, Wagenaar E, van Deemter L, Mol CA, Ottenhoff R, van der Lugt NM, van Roon MA and et al. (1993) Homozygous disruption of the murine *mdr2* P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* **75**(3): 451-462.
- Smith AJ, Timmermans-Hereijgers JL, Roelofsen B, Wirtz KW, van Blitterswijk WJ, Smit JJ, Schinkel AH and Borst P (1994) The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. *FEBS letters* **354**(3): 263-266.
- Smith AJ, van Helvoort A, van Meer G, Szabo K, Welker E, Szakacs G, Varadi A, Sarkadi B and Borst P (2000) MDR3 P-glycoprotein, a phosphatidylcholine translocase, transports several cytotoxic drugs and directly interacts with drugs as judged by interference with nucleotide trapping. *The Journal of biological chemistry* **275**(31): 23530-23539.
- Smith MT (2003) Mechanisms of troglitazone hepatotoxicity. *Chemical research in toxicology* **16**(6): 679-687.

- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC (1985) Measurement of Protein Using Bicinchoninic Acid. *Analytical biochemistry* **150**(1): 76-85.
- Somchit N, Hassim SM and Samsudin SH (2002) Itraconazole- and fluconazole-induced toxicity in rat hepatocytes: a comparative in vitro study. *Human & experimental toxicology* **21**(1): 43-48.
- Somchit N, Norshahida AR, Hasiah AH, Zuraini A, Sulaiman MR and Noordin MM (2004) Hepatotoxicity induced by antifungal drugs itraconazole and fluconazole in rats: a comparative in vivo study. *Human & experimental toxicology* **23**(11): 519-525.
- Speisky H and Cassels BK (1994) Boldo and boldine: an emerging case of natural drug development. *Pharmacol Res* **29**(1): 1-12.
- Stewart WW (1978) Functional connections between cells as revealed by dye-coupling with a highly fluorescent naphthalimide tracer. *Cell* **14**(3): 741-759.
- Stewart WW (1981) Lucifer dyes--highly fluorescent dyes for biological tracing. *Nature* **292**(5818): 17-21.
- Stieger B (2010) Role of the bile salt export pump, BSEP, in acquired forms of cholestasis. *Drug metabolism reviews* **42**(3): 437-445.
- Stieger B (2011) The role of the sodium-taurocholate cotransporting polypeptide (NTCP) and of the bile salt export pump (BSEP) in physiology and pathophysiology of bile formation. *Handbook of experimental pharmacology*(201): 205-259.
- Suzuki A, Andrade RJ, Bjornsson E, Lucena MI, Lee WM, Yuen NA, Hunt CM and Freston JW (2010) Drugs associated with hepatotoxicity and their reporting frequency of liver adverse events in Vigibase: unified list based on international collaborative work. *Drug safety* **33**(6): 503-522.
- Szakacs G, Varadi A, Ozvegy-Laczka C and Sarkadi B (2008) The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). *Drug discovery today* **13**(9-10): 379-393.
- Talwalkar JA, Soetikno RE, Carr-Locke DL and Berg CL (1999) Severe cholestasis related to itraconazole for the treatment of onychomycosis. *The American journal of gastroenterology* **94**(12): 3632-3633.
- Thompson RA, Isin EM, Li Y, Weidolf L, Page K, Wilson I, Swallow S, Middleton B, Stahl S, Foster AJ, Dolgos H, Weaver R and Kenna JG (2012) In vitro approach to assess the potential for risk of idiosyncratic adverse reactions caused by candidate drugs. *Chemical research in toxicology* **25**(8): 1616-1632.
- Trauner M, Fickert P, Halilbasic E and Moustafa T (2008) Lessons from the toxic bile concept for the pathogenesis and treatment of cholestatic liver diseases. *Wiener medizinische Wochenschrift* **158**(19-20): 542-548.

- Trauner M, Fickert P and Wagner M (2007) MDR3 (ABCB4) defects: a paradigm for the genetics of adult cholestatic syndromes. *Seminars in liver disease* **27**(1): 77-98.
- Trubiano JA, Crowe A, Worth LJ, Thursky KA and Slavin MA (2015) Putting CYP2C19 genotyping to the test: utility of pharmacogenomic evaluation in a voriconazole-treated haematology cohort. *The Journal of antimicrobial chemotherapy* **70**(4): 1161-1165.
- Uchida Y, Ohtsuki S, Kamiie J, Ohmine K, Iwase R and Terasaki T (2015) Quantitative targeted absolute proteomics for 28 human transporters in plasma membrane of Caco-2 cell monolayer cultured for 2, 3, and 4 weeks. *Drug metabolism and pharmacokinetics* **30**(2): 205-208.
- van Den Elsen JM, Kuntz DA, Hoedemaeker FJ and Rose DR (1999) Antibody C219 recognizes an alpha-helical epitope on P-glycoprotein. *Proceedings of the National Academy of Sciences of the United States of America* **96**(24): 13679-13684.
- van der Blik AM, Kooiman PM, Schneider C and Borst P (1988) Sequence of *mdr3* cDNA encoding a human P-glycoprotein. *Gene* **71**(2): 401-411.
- van Helvoort A, Smith AJ, Sprong H, Fritzsche I, Schinkel AH, Borst P and van Meer G (1996) MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* **87**(3): 507-517.
- Vavricka SR, Van Montfoort J, Ha HR, Meier PJ and Fattinger K (2002) Interactions of rifamycin SV and rifampicin with organic anion uptake systems of human liver. *Hepatology* **36**(1): 164-172.
- Vrins C, Vink E, Vandenberghe KE, Frijters R, Seppen J and Groen AK (2007) The sterol transporting heterodimer ABCG5/ABCG8 requires bile salts to mediate cholesterol efflux. *FEBS letters* **581**(24): 4616-4620.
- Wang JS, Zhu HJ, Markowitz JS, Donovan JL and DeVane CL (2006) Evaluation of antipsychotic drugs as inhibitors of multidrug resistance transporter P-glycoprotein. *Psychopharmacology* **187**(4): 415-423.
- Wegener T and Fintelmann V (1999) [Pharmacological properties and therapeutic profile of artichoke (*Cynara scolymus* L.)]. *Wiener medizinische Wochenschrift* **149**(8-10): 241-247.
- Wei G, Bergquist A, Broome U, Lindgren S, Wallerstedt S, Almer S, Sangfelt P, Danielsson A, Sandberg-Gertzen H, Loof L, Prytz H and Bjornsson E (2007) Acute liver failure in Sweden: etiology and outcome. *Journal of internal medicine* **262**(3): 393-401.
- Wlcek K and Stieger B (2014) ATP-binding cassette transporters in liver. *BioFactors* **40**(2): 188-198.
- Wu CP, Calcagno AM and Ambudkar SV (2008) Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: evaluation of current strategies. *Curr Mol Pharmacol* **1**(2): 93-105.
- Yang K, Guo C, Woodhead JL, St Claire RL, 3rd, Watkins PB, Siler SQ, Howell BA and Brouwer KL (2016) Sandwich-Cultured Hepatocytes as a Tool to Study Drug Disposition and Drug-Induced Liver Injury. *Journal of pharmaceutical sciences* **105**(2): 443-459.

- Yang K, Pfeifer ND, Kock K and Brouwer KL (2015) Species differences in hepatobiliary disposition of taurocholic acid in human and rat sandwich-cultured hepatocytes: implications for drug-induced liver injury. *The Journal of pharmacology and experimental therapeutics* **353**(2): 415-423.
- Yoshikado T, Takada T, Yamamoto T, Yamaji H, Ito K, Santa T, Yokota H, Yatomi Y, Yoshida H, Goto J, Tsuji S and Suzuki H (2011) Itraconazole-Induced Cholestasis: Involvement of the Inhibition of Bile Canalicular Phospholipid Translocator MDR3/ABCB4. *Molecular pharmacology* **79**(2): 241-250.
- Yuan L and Kaplowitz N (2013) Mechanisms of drug-induced liver injury. *Clinics in liver disease* **17**(4): 507-518, vii.
- Zagorova M, Prasnicka A, Kadova Z, Dolezelova E, Kazdova L, Cermanova J, Rozkydalova L, Hroch M, Mokry J and Micuda S (2015) Boldine attenuates cholestasis associated with nonalcoholic fatty liver disease in hereditary hypertriglyceridemic rats fed by high-sucrose diet. *Physiological research / Academia Scientiarum Bohemoslovaca* **64 Suppl 4**: S467-476.
- Zanger UM and Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacology & therapeutics* **138**(1): 103-141.
- Zhang D, Luo G, Ding X and Lu C (2012) Preclinical experimental models of drug metabolism and disposition in drug discovery and development *Acta Pharmaceutica Sinica B* **2**(6): 549–561.
- Zhang P, Tian X, Chandra P and Brouwer KL (2005) Role of glycosylation in trafficking of Mrp2 in sandwich-cultured rat hepatocytes. *Molecular pharmacology* **67**(4): 1334-1341.
- Zimmerman HJ (2000) Drug-induced liver disease. *Clinics in liver disease* **4**(1): 73-96, vi.

7 Appendix

7.1 Publications

Mahdi ZM, Synal-Hermanns U, Yoker A, Locher KP and Stieger B (2016). Role of Multidrug Resistance Protein 3 (MDR3) in Antifungal-Induced Cholestasis. *Molecular Pharmacology*. **90**(1): 23-34

Stieger B, **Mahdi ZM**, Jäger W (2017). Intestinal and Hepatocellular Transporters: Therapeutic Effects and Drug Interactions of Herbal Supplements. *Annual Review of Pharmacology and Toxicology*. [2016, Epub ahead of print]

7.2 Presentations at selected conferences

2015	Biomedical Transporter Conference, Lugano (poster)
2015	Day of Day of Clinical Research, University Hospital Zurich (poster)
2015	Deuel Conference on Lipids, Monterey, California (poster)
2014	7 th SFB35 Transmembrane transporters in health and disease (talk and poster)
2014	13 th Hepatobiliary and Gastrointestinal Research Retreat, Vulpera (talk)
2013	Biomedical Transporter Conference, St. Moritz (poster)
2013	Hepatobiliary Transport, Bile Acids and Liver Disease, Mariahof, Austria (talk)
2013	12 th Hepatobiliary and Gastrointestinal Research Retreat, Vulpera (talk)